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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Avi J. Ashkenazi, et al. Serial No.: 09/396,710 Filed: September 15, 1999 For: Apo-2 Receptor Antibodies	Group Art Unit: 1647 Examiner: Kaufman, C. Customer No:09157
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DECLARATION UNDER 37 C.F.R. 1.131

I, Avi J. Ashkenazi, hereby declare as follows:

1. I am the named inventor of the claimed subject matter of the above-identified patent application.
2. The above-identified patent application claims priority to application serial no. 08/857,216 filed with the Patent Office on May 15, 1997, and I am the named inventor in that priority application. A copy of my priority application serial no. 08/857,216 (hereinafter the "'216 application") is attached as Exhibit A.
3. All work described in the above-identified application and the '216 application was performed by me or on my behalf in the United States of America.
4. I have read and reviewed US Provisional Application 60/040,846 filed on March 17, 1997 (hereinafter the "'846 application") (a copy of which is attached as Exhibit B).
5. I have also read and reviewed US Provisional Application 60/054,021 filed on July 29, 1997 (hereinafter the "'021 application") (a copy of which is attached as Exhibit C).
6. The '216 application filed on my behalf on May 15, 1997 demonstrates both my conception of the claimed invention of the present application and a constructive reduction to practice of the invention.

7. Experiments performed by me or on my behalf relating to the identification and structural characterization of the Apo-2 receptor are described, for example, in Example 1 of the '216 application, pages 58-62. In *in vitro* binding assays, I found that the Apo-2 receptor extracellular domain binds the ligand known as Apo-2 ligand (the '216 application, e.g., pages 63, lines 9-35 - page 64, lines 1-6). In further *in vitro* assays, I also found that the Apo-2 receptor was capable of inducing cell death in transfected mammalian cells (the '216 application, page 64, lines 9-35 - page 65, lines 1 -13).

8. In the '216 application, agonist antibodies to the Apo-2 receptor are described. (See, e.g., Page 10, lines 3-5; Page 15, lines 7-10; Page 56, lines 21-23). More particularly, the '216 application discloses that an agonistic Apo-2 antibody may be employed to activate or stimulate apoptosis in mammalian cancer cells (Page 56, lines 21-23). Methods for making Apo-2 antibodies are described on pages 48-56 of the '216 application. Apoptotic activity in mammalian cells is described on, e.g., page 17, lines 1-12, of the '216 application.

9. The '216 application therefore demonstrates that agonist antibodies which bind Apo-2 receptor and stimulate apoptosis were conceived and constructively reduced to practice by the May 15, 1997 filing date of my patent application.

10. The '846 application was filed by Human Genome Sciences prior to the May 15, 1997, filing date of my patent application.

11. The '846 application does not provide information as to how the claimed sequences were identified or particularly, what materials or methods were used to identify the claimed sequences. In a scientific article published after the '846 application filing date (Pan et al., *Science*, 277:815-818 (1997)), the inventors of the '846 application indicate that the sequences were determined using database mining and human EST searching techniques.

12. The '846 application postulates that the disclosed polynucleotide sequences encode a putative "death domain"-containing receptor belonging to the Tumour Necrosis Factor (TNF) receptor superfamily. See, e.g., page 6 lines 31-33. The basis for that prediction appears to be certain homologies between the sequence described in the '846 application and the sequences of other members of the TNF receptor superfamily. The '846

application further postulates the activity or function of certain predicted polypeptide sequences, apparently on the basis of sequence homologies with some known TNF receptor family proteins. See, e.g., page 23, lines 20-29.

13. The '846 application at page 5, lines 8-13, and in Figure 2 illustrates the various similarities or homologies between the disclosed sequence (termed "DR5") and three other members of the TNF receptor family known at the time of the March 17, 1997 filing date; namely, TNFR1, Fas, and death domain containing receptor 3 (DR3). The '846 application states at page 6, lines 28-30 that the DR5 polypeptide shares the greatest degree of homology with those three known receptors. Overall, however such sequence homologies are strikingly low; the DR5 sequence is only about 19% identical to TNFR1, about 17% identical to Fas, and about 30% identical to DR3.

14. The '846 application at page 9, lines 13-18 further postulates that the DR5 sequence comprises a death domain comprising amino acids 324-391 shown in Figure 1. The '846 application acknowledges that this determination is a prediction based upon computer analysis and, therefore, is subject to variation. That postulated death domain, comprising amino acids 324-391 of the DR5 receptor, has very low homology to the respective death domains of TNFR1 (about 30%), Fas (about 19%), and DR3 (about 29%). The '846 application does not provide any further analysis regarding the identity or conservation of specific amino acids within the putative death domain, which were known to be crucial for activity of the death domain of TNFR1 (see, e.g., Table 2, Tartaglia et al Cell 74, 845-853 (1993); Fig 4B, Brojatsch et al, Cell 87, 845-855, (1996)).

15. The specification of the '846 application acknowledges that members of the TNF ligand or TNF receptor superfamily are known to have wide and divergent range of biological activities and functions. In particular, the '846 application states that the functions of ligands and receptors of the TNF superfamily are extremely diverse, and that ligands for members of the TNF receptor superfamily are "among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities and the transcriptional regulation of several genes." See, e.g., '846 application at pages 3 and 4.

16. The '846 application contains no experimental data characterizing the expression product(s) of the disclosed nucleotide sequences. In view of the relatively low homology of

the putative death domain described in the specification for the DR5 receptor to the death domain motifs of TNFR1, Fas and DR3, a person skilled in the art could not have reasonably determined that the DR5 polypeptide receptor could mediate or be involved in specific biological functions, such as apoptosis.

17. The '846 application, at page 6, lines 22-24, indicates that genes were identified in cDNA libraries of tissues of primary dendritic cells, endothelial tissue, spleen, chronic lymphocytic leukemia and human thymus stromal cells. A disclosure of such a wide expression pattern does not provide sufficient information to a person skilled in the art to suggest any specific activity or function for the molecule encoded by the disclosed sequence.

18. Instead of providing experimental data characterizing the function or activity of DR5, the '846 application relies on prophetic examples (see, e.g., prophetic expression in host cells such as *E. coli*, mammalian cells, baculovirus - Examples 1, 2, and 3; prophetic Northern Blot assay - Example 4; prophetic apoptosis assay - Example 5). The prophetic methods described in the '846 application may teach how to screen for a function of the putative receptor, but because of the acknowledged unpredictability of the functions associated with the diverse members of the TNF receptor superfamily, the function of the putative DR5 receptor could only reasonably have been determined until after the function had been observed or identified in an experimental assay.

19. The '846 application does not identify the actual ligands that specifically bind to the putative DR5 receptor. Instead, the '846 application provides a list of known TNF family ligands (see page 31, lines 4 to 9). The '846 application suggests that as many as nine different members of the TNF ligand superfamily that had been identified at the time of filing of the '846 application (page 1, lines 21-22) could function as ligands to the receptor. Subsequent disclosures reveal that many of these members of the TNF ligand superfamily do not, in fact, act as ligands to the putative DR5 receptor. In a subsequent filing by the inventors of the '846 application (i.e., the '021 application filed July 29, 1997), it is disclosed that Apo-2 ligand (or TRAIL) is a ligand for the DR5 receptor. That ligand, however, does not bind to any one of the TNFR1, Fas or DR3 receptors. In other words, the purported homology between the DR5, TNFR1, Fas and DR3 receptors reported in the '846 application was not predictive of, and could not have predicted, the actual ligand binding patterns of the DR5 receptor.

20. For at least these reasons, I believe the '846 application does not and cannot provide an adequate basis to determine the specific biological functions of the putative DR5 receptor disclosed in the '846 application. In particular, it is my opinion that a person of ordinary skill in this field would not be able to determine, in view of the relatively low homology of the putative death domain described in the specification for the DR5 receptor to the death domain motifs of TNFR1, Fas and DR3, that the polypeptide receptor could mediate specific biological functions, such as apoptosis. The functional complexity of the TNF receptor superfamily members that contain death domain motifs is further illustrated by the example of the low affinity NGF receptor (p75 NGFR, also called "neurotrophin receptor" or "NTR") known prior to the filing date of the '846 application. In Rabizadeh et al., Science, 261, 345-348 (1993), the authors teach that "expression of p75 NGFR induced neural cell death constitutively when p75 NGFR was unbound; binding by NGF or monoclonal antibody, however, inhibited cell death induced by p75 NGFR". In Chapman, FEBS Lett., 374, 216-220 (1995), the author states that "Unlike TNFR-1 and Fas, cell death induced by NTR (namely p75 NGFR) is reversed rather than caused by ligand binding". Thus, this death domain-containing member of the TNF receptor superfamily known at the time acted differently than others in the family in that its apoptosis inducing activity is switched "off", not "on", by ligand or antibody binding. Therefore, the mere presence of a death domain related sequence does not enable one skilled in the art to reasonably predict how a receptor will function.

21. I also note that there is no data provided in the '846 application characterizing any expression product of the DR5 sequence. Given the acknowledged diversity of biological functions and ligand binding patterns of the members of TNF receptor superfamily, it is my opinion that the absence of any data characterizing in any manner any expression product of the DR5 sequence in the '846 application renders the disclosure of that application insufficient to serve as a basis for determining the specific biological functions of the expression product of the DR5 sequence.

22. In light of the above observations and facts, it is my opinion that with respect to the '846 application one skilled in the art would not reasonably understand how to make and use antibodies raised against the DR5 receptor having specific biological functions (e.g., agonist or antagonist) that are mediated by the binding of such antibodies to the receptor.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

3/17/04
Date

Avi J Ashkenazi
Avi J. Ashkenazi, Ph.D.

PATENT DOCKET NO. P1101
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Apo-2 Receptor

FIELD OF THE INVENTION

The present invention relates generally to the identification, isolation, and recombinant production of novel polypeptides, designated herein as "Apo-2".

BACKGROUND OF THE INVENTION

Apoptosis or "Programmed Cell Death"

Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of apoptotic cell death have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)]. Increased levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic anemia, myocardial infarction, stroke, reperfusion injury, and toxin-induced liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *E1A*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been observed to have apoptosis-inducing activity [Thompson, supra].

TNF Family of Cytokines

Various molecules, such as tumor necrosis factor- α ("TNF- α "), tumor necrosis factor- β ("TNF- β " or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996)]. Among these molecules, TNF- α , TNF- β , CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF- α and TNF- β have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al., Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF- α is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory

Symposium on Programmed Cell Death, Abstr. No. 10, (1995)].

Mutations in the mouse Fas/Apo-1 receptor or ligand genes (called *lpr* and *gld*, respectively) have been associated with some autoimmune disorders, indicating that Apo-1 ligand may play a role 5 in regulating the clonal deletion of self-reactive lymphocytes in the periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata et al., Science, 267:1449-1456 (1995)]. Apo-1 ligand is also reported to induce post-stimulation apoptosis in 10 CD4-positive T lymphocytes and in B lymphocytes, and may be involved in the elimination of activated lymphocytes when their 15 function is no longer needed [Krammer et al., supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies specifically binding to the Apo-1 receptor have been reported to exhibit cell killing activity that is comparable to or similar to that of TNF- α [Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

TNF Family of Receptors

Induction of various cellular responses mediated by such TNF family cytokines is believed to be initiated by their binding to specific cell receptors. Two distinct TNF receptors of approximately 55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified 20 [Hohman et al., J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl. Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991] and human and mouse cDNAs corresponding to both receptor types have been isolated and 25 characterized [Loetscher et al., Cell, 61:351 (1990); Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023 (1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991); Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive polymorphisms have been associated with both TNF receptor genes 30 [see, e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs share the typical structure of cell surface receptors including extracellular, transmembrane and intracellular regions. The extracellular portions of both receptors are found naturally 35 also as soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990); and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A.,

87:8331 (1990)]. More recently, the cloning of recombinant soluble TNF receptors was reported by Hale et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1 and TNFR2) contains a repetitive amino acid sequence pattern of four cysteine-rich domains (CRDs) designated 1 through 4, starting from the NH₂-terminus. Each CRD is about 40 amino acids long and contains 4 to 6 cysteine residues at positions which are well conserved [Schall et al., supra; Loetscher et al., supra; Smith et al., supra; Nophar et al., supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2- amino acids from about 54 to about 97; CRD3- amino acids from about 98 to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2, CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about 55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4- amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435 (1993)]. The potential role of the CRDs in ligand binding is also described by Banner et al., supra.

A similar repetitive pattern of CRDs exists in several other cell-surface proteins, including the p75 nerve growth factor receptor (NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature, 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J., 8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063 (1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al., supral]. CRDs are also found in the soluble TNFR (sTNFR)-like T2 proteins of the Shope and myxoma poxviruses [Upton et al., Virology, 160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335 (1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of these sequences indicates that the positions of the cysteine residues are well conserved. These receptors are sometimes collectively referred to as members of the TNF/NGF receptor superfamily. Recent studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid insertion in

this domain [Yan, H. and Chao, M.V., J. Biol. Chem., 266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H. and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of about 60 amino acids, between its CRD4 and transmembrane region, which is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol., 41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973 (1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region is found in TNFR2 but not in TNFR1.

Itoh et al. disclose that the Apo-1 receptor can signal an apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh et al., supra]. Expression of the Apo-1 antigen has also been reported to be down-regulated along with that of TNFR1 when cells are treated with either TNF- α or anti-Apo-1 mouse monoclonal antibody [Krammer et al., supra; Nagata et al., supra]. Accordingly, some investigators have hypothesized that cell lines that co-express both Apo-1 and TNFR1 receptors may mediate cell killing through common signaling pathways [Id.].

The TNF family ligands identified to date, with the exception of lymphotoxin- α , are type II transmembrane proteins, whose C-terminus is extracellular. In contrast, the receptors in the TNF receptor (TNFR) family identified to date are type I transmembrane proteins. In both the TNF ligand and receptor families, however, homology identified between family members has been found mainly in the extracellular domain ("ECD"). Several of the TNF family cytokines, including TNF- α , Apo-1 ligand and CD40 ligand, are cleaved proteolytically at the cell surface; the resulting protein in each case typically forms a homotrimeric molecule that functions as a soluble cytokine. TNF receptor family proteins are also usually cleaved proteolytically to release soluble receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the TNFR family have been identified. In Marsters et al., Curr. Biol., 6:750 (1996), investigators describe a full length native sequence human polypeptide, called Apo-3, which exhibits similarity to the TNFR

family in its extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that it contains a cytoplasmic death domain sequence [see also Marsters et al., Curr. Biol., 6:1669 (1996)]. Apo-3 has also been referred to by other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science, 274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al., Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4 was reported to contain a cytoplasmic death domain capable of engaging the cell suicide apparatus. Pan et al. disclose that DR4 is believed to be a receptor for the ligand known as Apo-2 ligand or TRAIL.

The Apoptosis-Inducing Signaling Complex

As presently understood, the cell death program contains at least three important elements - activators, inhibitors, and effectors; in *C. elegans*, these elements are encoded respectively by three genes, *Ced-4*, *Ced-9* and *Ced-3* [Steller, Science, 267:1445 (1995); Chinnaiyan et al., Science, 275:1122-1126 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell; 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the transcription factor, NF- κ B [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found in several metazoan proteins that regulate apoptosis, namely, the Drosophila protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482 (1995)]. Using the yeast-two hybrid system, Raven et al. report the identification of protein, wsl-1, which binds to the TNFR1 death domain [Raven et al., Programmed Cell Death Meeting, September 20-24, 1995, Abstract at page 127; Raven et al., European Cytokine Network, 7:Abstr. 82 at page 210 (April-June 1996)]. The

wsl-1 protein is described as being homologous to TNFR1 (48% identity) and having a restricted tissue distribution. According to Raven et al., the tissue distribution of wsl-1 is significantly different from the TNFR1 binding protein, TRADD.

Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al.; Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the Ced-3-related protease, MACH α /FLICE (caspase 8), into the death signalling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)]. MACH α /FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1 β converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, ced-3, and to the mammalian IL-1-converting enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, crmA [Ray et al., Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol. Chem., 270:3255-3260 (1995)].

As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF- κ B [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF- κ B is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735

(1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF- κ B is complexed with members of the I κ B inhibitor family; upon inactivation of the I κ B in response to certain stimuli, released NF- κ B translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

5

For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

SUMMARY OF THE INVENTION

10 Applicants have identified cDNA clones that encode novel polypeptides, designated in the present application as "Apo-2." It is believed that Apo-2 is a member of the TNFR family; full-length native sequence human Apo-2 polypeptide exhibits some similarities to some known TNFRs, including a cytoplasmic death domain region.

15 Full-length native sequence human Apo-2 also exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats. Apo-2 polypeptide has been found to be capable of triggering caspase-dependent apoptosis and activating NF- κ B. Applicants surprisingly found that the soluble extracellular domain of Apo-2 binds Apo-2

20 ligand (Apo-2L) and can inhibit Apo-2 ligand function. It is presently believed that Apo-2 ligand can signal via at least two different receptors, DR4 and the newly described Apo-2 herein.

25 In one embodiment, the invention provides isolated Apo-2 polypeptide. In particular, the invention provides isolated native sequence Apo-2 polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). In other embodiments, the isolated Apo-2 polypeptide comprises at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising residues 1 to 411 of

30 Figure 1 (SEQ ID NO:1).

35 In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1).

35 In another embodiment, the invention provides an isolated

death domain sequence of Apo-2. Optionally, the isolated death domain sequence comprises amino acid residues 324 to 391 of Fig. 1 (SEQ ID NO:1).

5 In another embodiment, the invention provides chimeric molecules comprising Apo-2 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2 fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2 fused to a heterologous polypeptide or amino acid sequence, such as 10 an immunoglobulin sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2 polypeptide or a particular domain of Apo-2, or is complementary to 15 such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In one embodiment, the nucleic acid sequence is selected from:

20 (a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 411 (i.e., nucleotides 140-142 through 1370-1372), inclusive;

(b) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 182 (i.e., nucleotides 140-142 through 683-685), inclusive;

25 (c) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 54 to residue 182 (i.e., nucleotides 299-301 through 683-685), inclusive;

(d) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 324 to residue 391 30 (i.e., nucleotides 1109-1111 through 1310-1312), inclusive; or

(e) a sequence corresponding to the sequence of (a), (b), (c) or (d) within the scope of degeneracy of the genetic code.

In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the Apo-2 polypeptide or particular domain of Apo-2. A host cell comprising the vector 35

or the nucleic acid molecule is also provided. A method of producing Apo-2 is further provided.

In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an 5 agonistic, antagonistic or neutralizing antibody.

In another embodiment, the invention provides non-human, transgenic or knock-out animals.

A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies.

10

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence of a native sequence human Apo-2 cDNA and its derived amino acid sequence.

Figure 2A shows the derived amino acid sequence of a 15 native sequence human Apo-2 - the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.

Figure 2B shows an alignment and comparison of the death 20 domain sequences of native sequence human Apo-2, DR4, Apo-3/DR3, TNFR1, and Fas/Apo-1 (CD95). Asterisks indicate residues that are essential for death signaling by TNFR1 [Tartaglia et al., supra].

Figure 3 shows the interaction of the Apo-2 ECD with Apo-2L. Supernatants from mock-transfected 293 cells or from 293 cells 25 transfected with Flag epitope-tagged Apo-2 ECD were incubated with poly-His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag conjugated or Nickel conjugated agarose beads. The precipitated proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or 30 anti-Flag antibody.

Figure 4 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or by pRK5-based plasmids encoding Apo-2 and/or CrmA. Apoptosis was 35 assessed by morphology (A), DNA fragmentation (B), or by FACS (C-

E). Soluble Apo-2L was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody or Apo-2 ECD immunoadhesin or DR4 or TNFR1 immunoadhesins and added to HeLa cells. The cells were later analyzed for apoptosis (D). Dose-response analysis using Apo-2L with Apo-2 ECD immunoadhesin was also determined (E).

Figure 5 shows activation of NF- κ B by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF-alpha and assayed for NF- κ B activity. (C) HeLa cells were preincubated with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

Figure 6 shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

20 I. Definitions

The terms "Apo-2 polypeptide" and "Apo-2" when used herein encompass native sequence Apo-2 and Apo-2 variants (which are further defined herein). These terms encompass Apo-2 from a variety of mammals, including humans. The Apo-2 may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

A "native sequence Apo-2" comprises a polypeptide having the same amino acid sequence as an Apo-2 derived from nature. Thus, a native sequence Apo-2 can have the amino acid sequence of naturally-occurring Apo-2 from any mammal. Such native sequence Apo-2 can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2" specifically encompasses naturally-occurring truncated or secreted forms of the Apo-2 (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-

occurring allelic variants of the Apo-2. A naturally-occurring variant form of the Apo-2 includes an Apo-2 having an amino acid substitution at residue 410 in the amino acid sequence shown in Figure 1 (SEQ ID NO:1). In one embodiment of such naturally-
5 occurring variant form, the leucine residue at position 410 is substituted by a methionine residue. In Fig. 1 (SEQ ID NO:1), the amino acid residue at position 410 is identified as "Xaa" to indicate that the amino acid may, optionally, be either leucine or methionine. In Fig. 1 (SEQ ID NO:2), the nucleotide at position
10 1367 is identified as "W" to indicate that the nucleotide may be either adenine (A) or thymine (T) or uracil (U). In one embodiment of the invention, the native sequence Apo-2 is a mature or full-length native sequence Apo-2 comprising amino acids 1 to 411 of Fig. 1 (SEQ ID NO:1).

15 The "Apo-2 extracellular domain" or "Apo-2 ECD" refers to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than 0.5% of such domains. Optionally,
20 Apo-2 ECD will comprise amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1) or amino acid residues 1 to 182 of Fig. 1 (SEQ ID NO:1).

"Apo-2 variant" means a biologically active Apo-2 as defined below having at least about 80% amino acid sequence
25 identity with the Apo-2 having the deduced amino acid sequence shown in Fig. 1 (SEQ ID NO:1) for a full-length native sequence human Apo-2. Such Apo-2 variants include, for instance, Apo-2 polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID NO:1). Ordinarily, an Apo-2 variant will have at least about 80% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, and even more preferably at least about 95% amino acid sequence identity with the amino acid sequence of Fig. 1 (SEQ ID NO:1).

30 35 "Percent (%) amino acid sequence identity" with respect

to the Apo-2 sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the Apo-2 sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN™ or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising Apo-2, or a domain sequence thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the Apo-2. The tag polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning

cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the Apo-2 natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" Apo-2 nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the Apo-2 nucleic acid. An isolated Apo-2 nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 nucleic acid molecules therefore are distinguished from the Apo-2 nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 nucleic acid molecule includes Apo-2 nucleic acid molecules contained in cells that ordinarily express Apo-2 where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that

the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers single anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-Apo-2 antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain (e.g. "humanized" antibodies), or a light chain with a heavy chain, or a chain from one species with a chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')₂, and Fv), so long as they exhibit the desired biological activity. See, e.g. U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous

population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by
5 Kohler and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

"Humanized" forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')₂ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part,
10 humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the
15 humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human
20 immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.
25

"Biologically active" and "desired biological activity"
30 for the purposes herein mean having the ability to modulate

apoptosis (either in an agonistic or stimulating manner or in an antagonistic or blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*.

The terms "apoptosis" and "apoptotic activity" are used
5 in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function.
10 This activity can be determined and measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

The terms "treating," "treatment," and "therapy" as used
herein refer to curative therapy, prophylactic therapy, and
15 preventative therapy.

The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

20 II. Compositions and Methods of the Invention
The present invention provides newly identified and isolated Apo-2 polypeptides. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2 polypeptides disclosed herein, it is Applicants' present belief that Apo-2 is a member of the TNFR family.

30 A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared.

A. Preparation of Apo-2

The description below relates primarily to production of Apo-2 by culturing cells transformed or transfected with a vector containing Apo-2 nucleic acid. It is of course, contemplated that 5 alternative methods, which are well known in the art, may be employed to prepare Apo-2.

1. Isolation of DNA Encoding Apo-2

The DNA encoding Apo-2 may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 mRNA and 10 to express it at a detectable level. Accordingly, human Apo-2 DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage libraries of human pancreas and kidney cDNA described in Example 1. The Apo-2-encoding gene may also be obtained from a genomic library or by 15 oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the Apo-2 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by 20 it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding Apo-2 is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer:A 25 Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

A preferred method of screening employs selected oligonucleotide sequences to screen cDNA libraries from various 30 human tissues. Example 1 below describes techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like ^{32}P -labeled ATP, 35 biotinylation or enzyme labeling. Hybridization conditions,

including moderate stringency and high stringency, are provided in Sambrook et al., supra.

Nucleic acid having all the protein coding sequence may be obtained by screening selected cDNA or genomic libraries using 5 the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

10 Apo-2 variants can be prepared by introducing appropriate nucleotide changes into the Apo-2 DNA, or by synthesis of the desired Apo-2 polypeptide. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the Apo-2, such as changing the number or position of 15 glycosylation sites or altering the membrane anchoring characteristics.

Variations in the native full-length sequence Apo-2 or in various domains of the Apo-2 described herein, can be made, for example, using any of the techniques and guidelines for 20 conservative and non-conservative mutations set forth, for instance, in U.S. Pat. No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the Apo-2 that results in a change in the amino acid sequence of the Apo-2 as compared with the native sequence Apo-2. Optionally 25 the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the Apo-2 molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis 30 [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or 35 other known techniques can be performed on the cloned DNA to produce the Apo-2 variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence which are involved in the interaction with a particular ligand or receptor. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is the preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Once selected Apo-2 variants are produced, they can be contacted with, for instance, Apo-2L, and the interaction, if any, can be determined. The interaction between the Apo-2 variant and Apo-2L can be measured by an *in vitro* assay, such as described in the Examples below. While any number of analytical measurements can be used to compare activities and properties between a native sequence Apo-2 and an Apo-2 variant, a convenient one for binding is the dissociation constant K_d of the complex formed between the Apo-2 variant and Apo-2L as compared to the K_d for the native sequence Apo-2. Generally, a ≥ 3 -fold increase or decrease in K_d per substituted residue indicates that the substituted residue(s) is active in the interaction of the native sequence Apo-2 with the Apo-2L.

Optionally, representative sites in the Apo-2 sequence suitable for mutagenesis would include sites within the extracellular domain, and particularly, within one or both of the cysteine-rich domains. Such variations can be accomplished using the methods described above.

2. Insertion of Nucleic Acid into A Replicable Vector

The nucleic acid (e.g., cDNA or genomic DNA) encoding Apo-2 may be inserted into a replicable vector for further cloning

(amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is described below.

(i) Signal Sequence Component

The Apo-2 may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces* α -factor leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2.

(ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of Apo-2 DNA. However, the recovery of genomic DNA encoding Apo-2 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2 DNA.

(iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host

cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins,
5 e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al.,
10 J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)) or hygromycin [Sugden et al.,
15 Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Apo-2 nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely
20 adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes Apo-2.
25 Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of Apo-2 are synthesized from the amplified DNA. Other examples of amplifiable genes
30 include metallothionein-I and -II, adenosine deaminase, and
35

ornithine decarboxylase.

Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA comprising the expression vectors, such as the DNA encoding Apo-2. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Apo-2, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan. Similarly, Leu2-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6 μ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et al., Curr. Genet., **12**:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, **8**:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed [Fleer et al., Bio/Technology, **9**:968-975 (1991)].

10 (iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 nucleic acid sequence. Promoters are untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of particular nucleic acid sequence, such as the Apo-2 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 DNA.

30 Promoters suitable for use with prokaryotic hosts include the β -lactamase and lactose promoter systems [Chang et al., Nature, **275**:615 (1978); Goeddel et al., Nature, **281**:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., **8**:4057 (1980); EP 36,776], and hybrid promoters such as 35 the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, **80**:21-

25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding Apo-2 [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors 5 to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2.

Promoter sequences are known for eukaryotes. Virtually 10 all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an 15 AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase 20 [Hitzeman et al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)], such as enolase, 25 glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol 30 dehydrogenase 2, isocytchrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and 35 promoters for use in yeast expression are further described in EP 73,657. Yeast enhancers also are advantageously used with yeast promoters.

Apo-2 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and most preferably Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, from heat-shock promoters, and from the promoter normally associated with the Apo-2 sequence, provided such promoters are compatible with the host cell systems.

The early and late promoters of the SV40 virus are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication [Fiers et al., Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate early promoter of the human cytomegalovirus is conveniently obtained as a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360 (1982)]. A system for expressing DNA in mammalian hosts using the bovine papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446. A modification of this system is described in U.S. Patent No. 4,601,978 [See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA encoding immune interferon in monkey cells; Reyes et al., Nature, 297:598-601 (1982) on expression of human β -interferon cDNA in mouse cells under the control of a thymidine kinase promoter from herpes simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170 (1982) on expression of the human interferon β 1 gene in cultured mouse and rabbit cells; and Gorman et al., Proc. Natl. Acad. Sci. USA, 79:6777-6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells, HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long terminal repeat as a promoter].

(v) Enhancer Element Component

Transcription of a DNA encoding the Apo-2 of this

invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Enhancers are relatively 5 orientation and position independent, having been found 5' [Laimins et al., Proc. Natl. Acad. Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108 (1983)] to the transcription unit, within an intron [Banerji et al., Cell, 33:729 (1983)], as well as within the coding sequence itself [Osborne et al., Mol. Cell Bio., 10 4:1293 (1984)]. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the 15 cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. See also Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters. The enhancer may be spliced into the vector at a position 5' or 3' to the Apo-2 coding 20 sequence, but is preferably located at a site 5' from the promoter.

(vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for 25 the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA 30 encoding Apo-2.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques. Isolated plasmids or DNA fragments are cleaved, 35 tailored, and re-ligated in the form desired to generate the

plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxam et al., Methods in Enzymology, 65:499 (1980).

10 (viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying Apo-2 variants.

25 (ix) Suitable Exemplary Vertebrate Cell Vectors

Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 30 117,058.

3. Selection and Transformation of Host Cells

Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are not limited to eubacteria, such as Gram-negative or

Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

Suitable host cells for the expression of glycosylated Apo-2 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, e.g., Luckow et al., *Bio/Technology*, 6:47-55 (1988); Miller et al., in Genetic Engineering, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., *Nature*, 315:592-594 (1985)]. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Apo-2

can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen., 1:561 (1982)]. In addition, DNA segments isolated from the upstream region of the T-DNA 780 gene are capable of activating or increasing transcription levels of plant-expressible genes in recombinant DNA-containing plant tissue [EP 321,196 published 21 June 1989].

Propagation of vertebrate cells in culture (tissue culture) is also well known in the art [See, e.g., Tissue Culture, Academic Press, Kruse and Patterson, editors (1973)]. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al., Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

Host cells are transfected and preferably transformed with the above-described expression or cloning vectors for Apo-2 production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

Transfection refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in

fact expressed. Numerous methods of transfection are known to the ordinarily skilled artisan, for example, CaPO₄ and electroporation. Successful transfection is generally recognized when any indication of the operation of this vector occurs within the host cell.

5 Transformation means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal integrant. Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as 10 described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as 15 described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. In addition, plants may be transfected using ultrasound treatment as described in WO 91/00358 published 10 January 1991.

For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 20 52:456-457 (1978) is preferred. General aspects of mammalian cell host system transformations have been described in U.S. Pat. No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 25 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348- 30 352 (1988).

4. Culturing the Host Cells

Prokaryotic cells used to produce Apo-2 may be cultured in suitable media as described generally in Sambrook et al., supra. 35 The mammalian host cells used to produce Apo-2 may be

cultured in a variety of media. Examples of commercially available media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM", Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ("DMEM", Sigma). Any such media may be supplemented as necessary
5 with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art.
10 The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression,
15 and will be apparent to the ordinarily skilled artisan.

In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in
25 a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may
30 be employed, most commonly radioisotopes, and particularly ³²P. However, other techniques may also be employed, such as using biotin-modified nucleotides for introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels,
35 such as radionucleotides, fluorescers or enzymes. Alternatively,

antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a 5 surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of 10 cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With 15 immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, or luminescent labels.

Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence Apo-2 polypeptide or against a 20 synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to Apo-2 DNA and encoding a specific antibody epitope.

6. Purification of Apo-2 Polypeptide

Forms of Apo-2 may be recovered from culture medium or 25 from host cell lysates. If the Apo-2 is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X 100) or its extracellular domain may be released by enzymatic cleavage.

When Apo-2 is produced in a recombinant cell other than 30 one of human origin, the Apo-2 is free of proteins or polypeptides of human origin. However, it may be desired to purify Apo-2 from recombinant cell proteins or polypeptides to obtain preparations that are substantially homogeneous as to Apo-2. As a first step, the culture medium or lysate may be centrifuged to remove 35 particulate cell debris. Apo-2 thereafter is purified from

contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Apo-2 variants in which residues have been deleted, inserted, or substituted can be recovered in the same fashion as native sequence Apo-2, taking account of changes in properties occasioned by the variation. For example, preparation of an Apo-2 fusion with another protein or polypeptide, e.g., a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate purification; an immunoaffinity column containing antibody to the sequence can be used to adsorb the fusion polypeptide. Other types of affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native sequence Apo-2 may require modification to account for changes in the character of Apo-2 or its variants upon expression in recombinant cell culture.

7. Covalent Modifications of Apo-2 Polypeptides

Covalent modifications of Apo-2 are included within the scope of this invention. One type of covalent modification of the Apo-2 is introduced into the molecule by reacting targeted amino acid residues of the Apo-2 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of the Apo-2.

Derivatization with bifunctional agents is useful for crosslinking Apo-2 to a water-insoluble support matrix or surface for use in the method for purifying anti-Apo-2 antibodies, and

vice-versa. Derivatization with one or more bifunctional agents will also be useful for crosslinking Apo-2 molecules to generate Apo-2 dimers. Such dimers may increase binding avidity and extend half-life of the molecule *in vivo*. Commonly used crosslinking 5 agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-10 1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)-dithio]propioimidate yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen 15 bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl 20 residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 25 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

Another type of covalent modification of the Apo-2 polypeptide included within the scope of this invention comprises 30 altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence Apo-2, and/or adding one or more glycosylation sites that are not present in the native sequence 35 Apo-2.

Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the 5 recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either 10 of these tripeptide sequences in a polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the 15 attachment of one of the sugars N-acetylgalactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

Addition of glycosylation sites to the Apo-2 polypeptide 20 may be accomplished by altering the amino acid sequence such that 15 it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine residues to the native sequence Apo-2 (for O-linked glycosylation sites). The Apo-2 amino acid sequence may optionally 25 be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s) may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate 25 moieties on the Apo-2 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the 30 coupling mode used, the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These methods are described in WO 35 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2 polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. For instance, chemical deglycosylation by exposing the polypeptide to the compound trifluoromethanesulfonic acid, or an equivalent compound can result in the cleavage of most or all sugars except the linking sugar (N-acetylglucosamine or N-acetylgalactosamine), while leaving the polypeptide intact. Chemical deglycosylation is described by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be prevented by the use of the compound tunicamycin as described by Duskin et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the formation of protein-N-glycoside linkages.

Another type of covalent modification of Apo-2 comprises linking the Apo-2 polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

25 8. Apo-2 Chimeras

The present invention also provides chimeric molecules comprising Apo-2 fused to another, heterologous polypeptide or amino acid sequence.

In one embodiment, the chimeric molecule comprises a fusion of the Apo-2 with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the Apo-2. The presence of such epitope-tagged forms of the Apo-2 can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the Apo-2

to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

Various tag polypeptides and their respective antibodies
5 are well known in the art. Examples include the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus 10 glyccoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an α -tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. Once the tag polypeptide has been selected, an antibody thereto can be generated using the techniques disclosed herein.

Generally, epitope-tagged Apo-2 may be constructed and produced according to the methods described above. Epitope-tagged Apo-2 is also described in the Examples below. Apo-2-tag polypeptide fusions are preferably constructed by fusing the cDNA sequence encoding the Apo-2 portion in-frame to the tag polypeptide 25 DNA sequence and expressing the resultant DNA fusion construct in appropriate host cells. Ordinarily, when preparing the Apo-2-tag polypeptide chimeras of the present invention, nucleic acid encoding the Apo-2 will be fused at its 3' end to nucleic acid encoding the N-terminus of the tag polypeptide, however 5' fusions 30 are also possible. For example, a polyhistidine sequence of about 5 to about 10 histidine residues may be fused at the N- terminus or the C- terminus and used as a purification handle in affinity chromatography.

Epitope-tagged Apo-2 can be purified by affinity chromatography using the anti-tag antibody. The matrix to which 35

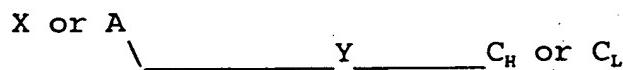
the affinity antibody is attached may include, for instance, agarose, controlled pore glass or poly(styrenedivinyl)benzene. The epitope-tagged Apo-2 can then be eluted from the affinity column using techniques known in the art.

5 In another embodiment, the chimeric molecule comprises an Apo-2 polypeptide fused to an immunoglobulin sequence. The chimeric molecule may also comprise a particular domain sequence of Apo-2, such as the extracellular domain sequence of native Apo-2 fused to an immunoglobulin sequence. This includes chimeras in
10 monomeric, homo- or heteromultimeric, and particularly homo- or heterodimeric, or -tetrameric forms; optionally, the chimeras may be in dimeric forms or homodimeric heavy chain forms. Generally, these assembled immunoglobulins will have known unit structures as represented by the following diagrams.

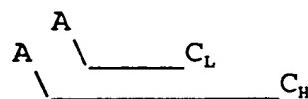
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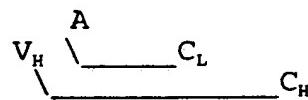
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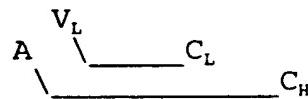
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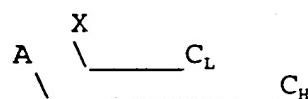
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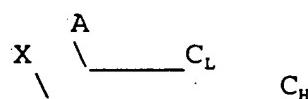
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A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in serum. In the case of multimers, each four chain unit may be the same or different.

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The following diagrams depict some exemplary monomer, homo- and heterodimer and homo- and heteromultimer structures. These diagrams are merely illustrative, and the chains of the

multimers are believed to be disulfide bonded in the same fashion as native immunoglobulins.

monomer: A C_L or C_H

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homodimer:

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heterodimer:

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homotopy

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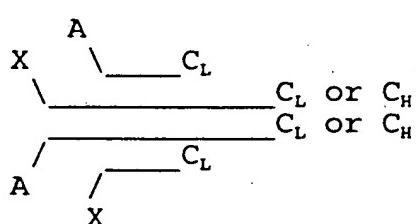
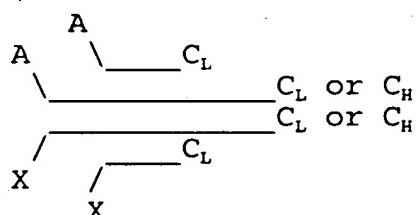
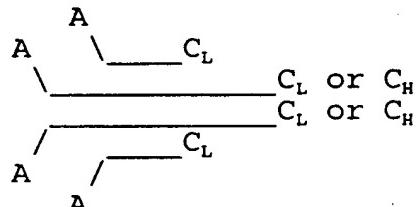
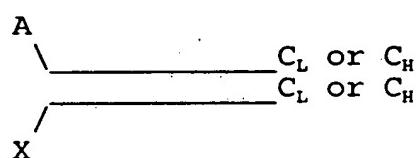
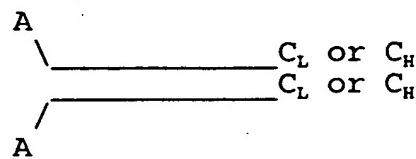
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and

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In the foregoing diagrams, "A" means an Apo-2 sequence or an Apo-2 sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a

portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such a pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon- γ) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and V_L , V_H , C_L and C_H represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an Apo-2 sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain from an antibody of one species is substituted for the variable domain of another species. See, for example, EP 0 125 023; EP 173,494; Munro, Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May 1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855 (1984); Morrison et al., Science, 229:1202-1207 (1985); Boulian et al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature, 337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

Alternatively, the chimeric molecules may be constructed

as follows. The DNA including a region encoding the desired sequence, such as an Apo-2 and/or TNFR sequence, is cleaved by a restriction enzyme at or proximal to the 3' end of the DNA encoding the immunoglobulin-like domain(s) and at a point at or near the DNA 5 encoding the N-terminal end of the Apo-2 or TNFR polypeptide (where use of a different leader is contemplated) or at or proximal to the N-terminal coding region for TNFR (where the native signal is employed). This DNA fragment then is readily inserted proximal to DNA encoding an immunoglobulin light or heavy chain constant region 10 and, if necessary, the resulting construct tailored by deletional mutagenesis. Preferably, the Ig is a human immunoglobulin when the chimeric molecule is intended for *in vivo* therapy for humans. DNA encoding immunoglobulin light or heavy chain constant regions is known or readily available from cDNA libraries or is synthesized. 15 See for example, Adams et al., Biochemistry, 19:2711-2719 (1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al., Proc. Natl. Acad. Sci. USA, 77:6027-6031 (1980); Rice et al., Proc. Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-288 (1982); and Morrison et al., Ann. Rev. 20 Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in publications concerning the preparation of immunoadhesins. Immunoadhesins in general, and CD4-Ig fusion molecules specifically are disclosed in WO 89/02922, published 6 April 1989). Molecules 25 comprising the extracellular portion of CD4, the receptor for human immunodeficiency virus (HIV), linked to IgG heavy chain constant region are known in the art and have been found to have a markedly longer half-life and lower clearance than the soluble extracellular portion of CD4 [Capon et al., supra; Byrn et al., Nature, 344:667 30 (1990)]. The construction of specific chimeric TNFR-IgG molecules is also described in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991); Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439)].

B. Therapeutic and Non-therapeutic Uses for Apo-2

Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric molecules containing the extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF- κ B induction by Apo-2L or by another ligand that Apo-2 binds to.

The Apo-2 of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 is present in the cell type(s) being evaluated. Apo-2 nucleic acid will also be useful for the preparation of Apo-2 by the recombinant techniques described herein.

The isolated Apo-2 may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 may be prepared. Apo-2 preparations are also useful in generating antibodies, as standards in assays for Apo-2 (e.g., by labeling Apo-2 for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.

Nucleic acids which encode Apo-2 or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal

(e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding Apo-2 or an appropriate sequence thereof (such as Apo-2-IgG) can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding Apo-2. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for Apo-2 transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding Apo-2 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding Apo-2. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with excessive apoptosis. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition. In another embodiment, transgenic animals that carry a soluble form of Apo-2 such as the Apo-2 ECD or an immunoglobulin chimera of such form could be constructed to test the effect of chronic neutralization of Apo-2L, a ligand of Apo-2.

Alternatively, non-human homologues of Apo-2 can be used to construct an Apo-2 "knock out" animal which has a defective or altered gene encoding Apo-2 as a result of homologous recombination between the endogenous gene encoding Apo-2 and altered genomic DNA encoding Apo-2 introduced into an embryonic cell of the animal. For example, cDNA encoding Apo-2 can be used to clone genomic DNA encoding Apo-2 in accordance with established techniques. A

portion of the genomic DNA encoding Apo-2 can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, *Cell*, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li et al., *Cell*, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the Apo-2 polypeptide, including for example, development of tumors.

25 C. Anti-Apo-2 Antibody Preparation

The present invention further provides anti-Apo-2 antibodies. Antibodies against Apo-2 may be prepared as follows. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

30 1. Polyclonal Antibodies

The Apo-2 antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or

adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a Apo-2-IgG fusion protein or chimeric molecule (including an Apo-2 ECD-IgG fusion protein). Cells expressing Apo-2 at their surface may also be employed. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins which may be employed include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. An aggregating agent such as alum may also be employed to enhance the mammal's immune response. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation. The mammal can then be bled, and the serum assayed for antibody titer. If desired, the mammal can be boosted until the antibody titer increases or plateaus.

20 2. Monoclonal Antibodies

The Apo-2 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, supra. In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized (such as described above) with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

30 The immunizing agent will typically include the Apo-2 polypeptide or a fusion protein thereof. An example of a suitable immunizing agent is a Apo-2-IgG fusion protein or chimeric molecule. Cells expressing Apo-2 at their surface may also be employed. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or

lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against Apo-2. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are

known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody

of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art. For instance, digestion can be performed using papain. Examples of papain digestion are described in WO 94/29348 published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of antibodies typically produces two identical antigen binding fragments, called Fab fragments, each with a single antigen binding site, and a residual Fc fragment. Pepsin treatment yields an $F(ab'), fragment that has two antigen combining sites and is still capable of cross-linking antigen.$

The Fab fragments produced in the antibody digestion also contain the constant domains of the light chain and the first constant domain (CH_1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH_1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. $F(ab'), antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.$

3. Humanized Antibodies

The Apo-2 antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric

immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')₂, or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins
5 (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human
10 immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two,
15 variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an
20 immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Reichmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed
25 following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized"
30 antibodies are chimeric antibodies (U.S. Patent No. 4,816,567),
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wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues 5 are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important in order to reduce antigenicity. According to the "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human framework (FR) for 10 the humanized antibody [Sims et al., J. Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901 (1987)]. Another method 15 uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci. USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of 25 analysis of the parental sequences and various conceptual humanized products using three dimensional models of the parental and humanized sequences. Three dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display 30 probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate 35 immunoglobulin to bind its antigen. In this way, FR residues can

be selected and combined from the consensus and import sequence so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding [see, WO 94/04679 published 3 March 5 1994].

Transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production can be employed. For example, it has been described that the homozygous deletion of the antibody heavy chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition 10 of endogenous antibody production. Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci. USA, 90:2551-255 15 (1993); Jakobovits et al., Nature, 362:255-258 (1993); Brugermann et al., Year in Immuno., 7:33 (1993)]. Human antibodies can also be produced in phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 20 (1991)]. The techniques of Cote et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cote et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)]. 25

4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other 30 antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have 35

different specificities [Millstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has
5 the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

According to a different and more preferred approach,
10 antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH₂, and CH₃ regions. It is preferred to have
15 the first heavy-chain constant region (CH₁) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable
20 host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all
25 three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy
30 chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in
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only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 5 121:210 (1986).

5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for 10 example, been proposed to target immune system cells to unwanted cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the 15 antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptoputyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

20 D. Therapeutic and Non-therapeutic Uses for Apo-2 Antibodies

The Apo-2 antibodies of the invention have therapeutic utility. Agonistic Apo-2 antibodies, for instance, may be employed 25 to activate or stimulate apoptosis in cancer cells. Alternatively, antagonistic antibodies may be used to block excessive apoptosis (for instance in neurodegenerative disease) or to block potential autoimmune/inflammatory effects of Apo-2 resulting from NF- κ B activation.

Apo-2 antibodies may further be used in diagnostic assays 30 for Apo-2, e.g., detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-35 158]. The antibodies used in the diagnostic assays can be labeled

with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^3H , ^{14}C , ^{32}P , ^{35}S , or ^{125}I , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

Apo-2 antibodies also are useful for the affinity purification of Apo-2 from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-2, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 from the antibody.

E. Kits Containing Apo-2 or Apo-2 Antibodies

In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is Apo-2 or an Apo-2 antibody. The label

on the container indicates that the composition is used for a specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

5 The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

10 *****
The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

15 All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

20 All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout 25 the specification, by ATCC accession numbers is the American Type Culture Collection, Rockville, Maryland.

EXAMPLE 1

Isolation of cDNA clones Encoding Human Apo-2

30 Expressed sequence tag (EST) DNA databases (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) were searched and an EST was identified which showed homology to the death domain of the Apo-3 receptor [Marsters et al., Curr. Biol., 6:750 (1996)]. Human pancreas and kidney lgt10 bacteriophage cDNA libraries (both purchased from Clontech) were ligated into pRK5 vectors as follows.

Reagents were added together and incubated at 16°C for 16 hours: 5X
T4 ligase buffer (3 ml); pRK5, Xhol, NotI digested vector, 0.5 mg,
1 ml); cDNA (5 ml) and distilled water (6 ml). Subsequently,
additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) were
5 added and the entire reaction was extracted through
phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was
removed, collected and diluted into 5M NaCl (10 ml) and absolute
ethanol (-20°C, 250 ml). This was then centrifuged for 20 minutes
10 at 14,000 x g, decanted, and the pellet resuspended into 70%
ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g.
The DNA pellet was then dried in a speedvac and eluted into
distilled water (3 ml) for use in the subsequent procedure.

The ligated cDNA/pRK5 vector DNA prepared previously was
chilled on ice to which was added electrocompetent DH10B bacteria
15 (Life Tech., 20 ml). The bacteria vector mixture was then
electroporated as per the manufacturers recommendation.
Subsequently SOC media (1 ml) was added and the mixture was
incubated at 37°C for 30 minutes. The transformants were then
20 plated onto 20 standard 150 mm LB plates containing ampicillin and
incubated for 16 hours (37°C) to allow the colonies to grow.
Positive colonies were then scraped off and the DNA isolated from
the bacterial pellet using standard CsCl-gradient protocols.

An enriched 5'-cDNA library was then constructed to
obtain a bias of cDNA fragments which preferentially represents the
25 5' ends of cDNA's contained within the library. 10 mg of the
pooled isolated full-length library plasmid DNA (41 ml) was
combined with Not I restriction buffer (New England Biolabs, 5 ml)
and Not I (New England Biolabs, 4 ml) and incubated at 37°C for one
hour. The reaction was extracted through phenol:chloroform:isoamyl
30 alcohol (25:24:1, 50 ml), the aqueous phase removed, collected and
resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150
ml). This was then centrifuged for 20 minutes at 14,000 x g,
decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged
again for 2 minutes at 14,000 x g. The supernatant was then
35 removed, the pellet dried in a speedvac and resuspended in

distilled water (10 ml).

The following reagents were brought together and incubated at 37°C for 2 hours: distilled water (3 ml); linearized DNA library (1 mg, 1 ml); Ribonucleotide mix (Invitrogen, 10 ml); transcription buffer (Invitrogen, 2 ml) and Sp6 enzyme mix. The reaction was then extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml) and the aqueous phase was removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml) and centrifuged for 20 minutes at 14,000 x g. The pellet was then decanted and resuspended in 70% ethanol (0.5 ml), centrifuged again for 2 minutes at 14,000 x g, decanted, dried in a speedvac and resuspended into distilled water (10 ml).

The following reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (Life Tech., 3 ml); pRK5 Cla-Sal digested vector, 0.5 mg, 1 ml); cDNA (5 ml); distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) was added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 100 ml). The aqueous phase was removed, collected and diluted by 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml) and centrifuged for 20 minutes at 14,000 x g. The DNA pellet was decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was removed and the residue pellet was dried in a speedvac and resuspended in distilled water (3ml). The ligated cDNA/pSST-amy.1 vector DNA was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as recommended by the manufacturer. Subsequently, SOC media (Life Tech., 1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C). Positive colonies were scraped off the plates and the DNA was isolated from the bacterial pellet using standard protocols, e.g. CsCl-gradient.

The cDNA libraries were screened by hybridization with a

synthetic oligonucleotide probe:

GGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGGCA
GCGGG (SEQ ID NO:3) based on the EST.

Three cDNA clones were sequenced in entirety. The overlapping coding regions of the cDNAs were identical except for codon 410 (using the numbering system for Fig. 1); this position encoded a leucine residue (TTG) in both pancreatic cDNAs, and a methionine residue (ATG) in the kidney cDNA, possibly due to polymorphism.

The entire nucleotide sequence of Apo-2 is shown in Figure 1 (SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as ATCC _____, as indicated below) contains a single open reading frame with an apparent translational initiation site at nucleotide positions 140-142 [Kozak et al., supra] and ending at the stop codon found at nucleotide positions 1373-1375 (Fig. 1; SEQ ID NO:2). The predicted polypeptide precursor is 411 amino acids long, a type I transmembrane protein, and has a calculated molecular weight of approximately 45 kDa. Hydropathy analysis (not shown) suggested the presence of a signal sequence (residues 1-53), followed by an extracellular domain (residues 54-182), a transmembrane domain (residues 183-208), and an intracellular domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid sequence analysis of Apo-2-IgG expressed in 293 cells showed that the mature polypeptide starts at amino acid residue 54, indicating that the actual signal sequence comprises residues 1-53.

TNF receptor family proteins are typically characterized by the presence of multiple (usually four) cysteine-rich domains in their extracellular regions -- each cysteine-rich domain being approximately 45 amino acids long and containing approximately 6, regularly spaced, cysteine residues. Based on the crystal structure of the type 1 TNF receptor, the cysteines in each domain typically form three disulfide bonds in which usually cysteines 1 and 2, 3 and 5, and 4 and 6 are paired together. Like DR4, Apo-2 contains two extracellular cysteine-rich pseudorepeats (Fig. 2A), whereas other identified mammalian TNFR family members contain

three or more such domains [Smith et al., *Cell*, **76**:959 (1994)].

The cytoplasmic region of Apo-2 contains a death domain (amino acid residues 324-391 shown in Fig. 1; see also Fig. 2A) which shows significantly more amino acid sequence identity to the death domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%); or Apo-3/DR3 (29%) (Fig. 2B). Four out of six death domain amino acids that are required for signaling by TNFR1 [Tartaglia et al., *supra*] are conserved in Apo-2 while the other two residues are semi-conserved (see Fig. 2B).

Based on an alignment analysis (using the ALIGN™ computer program) of the full-length sequence, Apo-2 shows more sequence identity to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1 (19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP) (29%).

EXAMPLE 2

A. Expression of Apo-2 ECD

A soluble extracellular domain (ECD) fusion construct was prepared. An Apo-2 ECD (amino acid residues 1-184 shown in Figure 1) was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma). (The Apo-2 ECD construct included residues 183 and 184 shown in Figure 1 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region). The Flag epitope-tagged molecule was then inserted into pRK5, and expressed by transient transfection into human 293 cells (ATCC CRL 1573).

After a 48 hour incubation, the cell supernatants were collected and either used directly for co-precipitation studies (see Example 3) or subjected to purification of the Apo-2 ECD-Flag by affinity chromatography on anti-Flag agarose beads, according to manufacturer's instructions (Sigma).

B. Expression of Apo-2 ECD as an Immunoadhesin

A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the

hinge and Fc region of human immunoglobulin G, heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra.

5. EXAMPLE 3

10 Immunoprecipitation Assay Showing Binding Interaction
Between Apo-2 and Apo-2 Ligand

To determine whether Apo-2 and Apo-2L interact or associate with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described in Example 2 above) (5 ml) were incubated with 5 µg poly-histidine-tagged soluble Apo-2L [Pitti et al., supra] for 30 minutes at room temperature and then analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using 25 µl anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4° C, the beads were spun down and washed four times in phosphate buffered saline (PBS). By using anti-Flag agarose, the Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-agarose, the Apo-2 ECD was precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide gels, and then detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2 µg/ml) as described in Marsters et al., J. Biol. Chem., (1997).

30 The results, shown in Figure 3, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

The binding interaction was further analyzed by purifying Apo-2 ECD from the transfected 293 cell supernatants with anti-Flag beads (see Example 2) and then analyzing the samples on a BIACORE™ instrument. The BIACORE™ analysis indicated a dissociation

constant (K_d) of about 1 nM. BIACORE™ analysis also showed that the Apo-2 ECD is not capable of binding other apoptosis-inducing TNF family members, namely, TNF-alpha (Genentech, Inc., Pennica et al., *Nature*, 312:712 (1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis Biochemicals). The data thus shows
5 that Apo-2 is a specific receptor for Apo-2L.

EXAMPLE 4

Induction of Apoptosis by Apo-2

Because death domains can function as oligomerization interfaces, over-expression of receptors that contain death domains may lead to activation of signaling in the absence of ligand [Frazer et al., *supra*, Nagata et al., *supra*]. To determine whether Apo-2 was capable of inducing cell death, human 293 cells or HeLa cells (ATCC CCL 2.2) were transiently transfected by calcium phosphate precipitation (293 cells) or electroporation (HeLa cells) with a pRK5 vector or pRK5-based plasmids encoding Apo-2 and/or CrmA. When applicable, the total amount of plasmid DNA was adjusted by adding vector DNA. Apoptosis was assessed 24 hours after transfection by morphology (Fig. 4A); DNA fragmentation (Fig. 4B); or by FACS analysis of phosphatydilserine exposure (Fig. 4C) as described in Marsters et al., *Curr. Biol.*, 6:1669 (1996). As shown in Figs. 4A and 4B, the Apo-2 transfected 293 cells underwent marked apoptosis.
10
15
20
25

For samples assayed by FACS, the HeLa cells were co-transfected with pRK5-CD4 as a marker for transfection and apoptosis was determined in CD4-expressing cells; FADD was co-transfected with the Apo-2 plasmid; the data are means \pm SEM of at least three experiments, as described in Marsters et al., *Curr. Biol.*, 6:1669 (1996). The caspase inhibitors, DEVD-fmk (Enzyme Systems) or z-VAD-fmk (Research Biochemicals Intl.) were added at 200 μ M at the time of transfection. As shown in Fig. 4C, the caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis induction by Apo-2, indicating the involvement of Ced-3-like proteases in this response.
30
35

FADD is an adaptor protein that mediates apoptosis activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., supra], but does not appear necessary for apoptosis induction by Apo-2L [Marsters et al., supra] or by DR4 [Pan et al., supra]. A 5 dominant-negative mutant form of FADD, which blocks apoptosis induction by CD95, TNFR1, or Apo-3/DR3 [Frazer et al., supra; Nagata et al., supra; Chinnayian et al., supra] did not inhibit apoptosis induction by Apo-2 when co-transfected into HeLa cells with Apo-2 (Fig. 4C). These results suggest that Apo-2 signals 10 apoptosis independently of FADD. Consistent with this conclusion, a glutathione-S-transferase fusion protein containing the Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and translated FADD (data not shown).

15

EXAMPLE 5

Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

Soluble Apo-2L (0.5 μ g/ml, prepared as described in Pitti et al., supra) was pre-incubated for 1 hour at room temperature with PBS buffer or affinity-purified Apo-2 ECD (5 μ g/ml) together 20 with anti-Flag antibody (Sigma) (1 μ g/ml) and added to HeLa cells. After a 5 hour incubation, the cells were analyzed for apoptosis by FACS (as above) (Fig. 4D).

Apo-2L induced marked apoptosis in HeLa cells, and the soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 4D), confirming a specific interaction between Apo-2L and Apo-2. Similar results were obtained with the Apo-2 ECD immunoadhesin (Fig. 4D). Dose-response analysis showed half-maximal inhibition at approximately 0.3 nM Apo-2 immunoadhesin (Fig. 4E).

30

EXAMPLE 6

Activation of NF- κ B by Apo-2

An assay was conducted to determine whether Apo-2 activates NF- κ B.

HeLa cells were transfected with pRK5 expression plasmids 35 encoding full-length native sequence Apo-2, DR4 or Apo-3 and

harvested 24 hours after transfection. Nuclear extracts were prepared and 1 μ g of nuclear protein was reacted with a 32 P-labelled NF- κ B-specific synthetic oligonucleotide probe

ATCAGGGACTTCCGCTGGGGACTTCCG (SEQ ID NO:4) [see, also, MacKay et al., J. Immunol., 153:5274-5284 (1994)], alone or together with a 50-fold excess of unlabelled probe, or with an irrelevant 32 P-labelled synthetic oligonucleotide

AGGATGGGAAGTGTGTGATATCCTTGAT (SEQ ID NO:5). In some samples, antibody to p65/RelA subunits of NF- κ B (1 μ g/ml; Santa Cruz Biotechnology) was added. DNA binding was analyzed by an electrophoretic mobility shift assay as described by Hsu et al., supra; Marsters et al., supra, and MacKay et al., supra.

The results are shown in Fig. 5. As shown in Fig. 5A, upon transfection into HeLa cells, both Apo-2 and DR4 induced significant NF- κ B activation as measured by the electrophoretic mobility shift assay; the level of activation was comparable to activation observed for Apo-3/DR3. Antibody to the p65/RelA subunit of NF- κ B inhibited the mobility of the NF- κ B probe, implicating p65 in the response to all 3 receptors.

An assay was also conducted to determine if Apo-2L itself can regulate NF- κ B activity. HeLa cells or MCF7 cells (human breast adenocarcinoma cell line, ATCC HTB 22) were treated with PBS buffer, soluble Apo-2L (Pitti et al., supra) or TNF-alpha (Genentech, Inc., see Pennica et al., Nature, 312:721 (1984)) (1 μ g/ml) and assayed for NF- κ B activity as above. The results are shown in Fig. 5B. The Apo-2L induced a significant NF- κ B activation in the treated HeLa cells but not in the treated MCF7 cells; the TNF-alpha induced a more pronounced activation in both cell lines. Several studies have disclosed that NF- κ B activation by TNF can protect cells against TNF-induced apoptosis [Nagata, supra].

The effects of a NF- κ B inhibitor, ALLN (N-acetyl-Leu-Leu-norleucinal) and a transcription inhibitor, cyclohexamide, were also tested. The HeLa cells (plated in 6-well dishes) were preincubated with PBS buffer, ALLN (Calbiochem) (40 μ g/ml) or

cyclohexamide (Sigma) (50 µg/ml) for 1 hour before addition of Apo-2L (1 µg/ml). After a 5 hour incubation, apoptosis was analyzed by FACS (see Fig. 5C).

The results are shown in Fig. 5C. Both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis in the HeLa cells. The data indicates that Apo-2L can induce protective NF-κB-dependent genes. The data also indicates that Apo-2L is capable of activating NF-κB in certain cell lines and that both Apo-2 and DR4 may mediate that function.

10

EXAMPLE 7

Northern Blot Analysis

Expression of Apo-2 mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a 4.6 kilobase ³²P-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

As shown in Fig. 6, a predominant mRNA transcript of approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, kidney, skeletal muscle, placenta, and heart. Several adult tissues that express Apo-2, e.g., PBL, ovary, and spleen, have been shown previously to express DR4 [Pan et al., supra], however, the relative levels of expression of each receptor mRNA appear to be different.

EXAMPLE 8

Chromosomal Localization of the Apo-2 gene

Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was 5 performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al., Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to the marker D8S481, with an LOD of 10 11.05; D8S481 is linked in turn to D8S2055, which maps to human chromosome 8p21. A similar analysis of DR4 showed that DR4 is linked to the marker D8S2127 (with an LOD of 13.00), which maps also to human chromosome 8p21.

To Applicants' present knowledge, to date, no other 15 member of the TNFR gene family has been located to chromosome 8.

* * * * *

Deposit of Material

The following materials have been deposited with the
American Type Culture Collection, 12301 Parklawn Drive, Rockville,
5 MD, USA (ATCC) :

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
pRK5-Apo-2	_____	_____

10 This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance
15 of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the
20 public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the
25 Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

30 The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

35 The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the

invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the
5 scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific
10 illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

15

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT: Ashkenazi, Avi J.

(ii) TITLE OF INVENTION: Apo-2 RECEPTOR

(iii) NUMBER OF SEQUENCES: 5

10 (iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: Genentech, Inc.
- (B) STREET: 460 Point San Bruno Blvd
- (C) CITY: South San Francisco
- 15 (D) STATE: California
- (E) COUNTRY: USA
- (F) ZIP: 94080

(v) COMPUTER READABLE FORM:

- 20 (A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk
- (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: WinPatin (Genentech)

25 (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:
- (B) FILING DATE: 15-May-1997
- (C) CLASSIFICATION:

30 (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Marschang, Diane L.
- (B) REGISTRATION NUMBER: 35,600
- (C) REFERENCE/DOCKET NUMBER: P1101

35 (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: 415/225-5416
(B) TELEFAX: 415/952-9881
(C) TELEX: 910/371-7168

5 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 411 amino acids
(B) TYPE: Amino Acid
10 (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg

15 1 5 10 15

Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala Arg Pro

20 20 25 30

20 Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val Val Ala Ala Val

35 35 40 45

Leu Leu Leu Val Ser Ala Glu Ser Ala Leu Ile Thr Gln Gln Asp

50 55 60

25 Leu Ala Pro Gln Gln Arg Ala Ala Pro Gln Gln Lys Arg Ser Ser

65 70 75

Pro Ser Glu Gly Leu Cys Pro Pro Gly His His Ile Ser Glu Asp

30 80 85 90

Gly Arg Asp Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr

95 100 105

35 His Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys Asp

	110	115	120
	Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr Arg Asn Thr		
	125	130	135
5			
	Val Cys Gln Cys Glu Glu Gly Thr Phe Arg Glu Glu Asp Ser Pro		
	140	145	150
	Glu Met Cys Arg Lys Cys Arg Thr Gly Cys Pro Arg Gly Met Val		
10	155	160	165
	Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His		
	170	175	180
15	Lys Glu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val		
	185	190	195
	Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys		
	200	205	210
20			
	Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Asp		
	215	220	225
	Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp		
25	230	235	240
	Asn Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val		
	245	250	255
30	Pro Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly		
	260	265	270
	Val Asn Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro		
	275	280	285
35			

Ala Glu Ala Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala
290 295 300

Asn Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp
5 305 310 315

Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg
320 325 330

10 Lys Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu
335 340 345

Ala Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp
350 355 360

15 Val Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp
365 370 375

Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu
20 380 385 390

Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn
395 400 405

25 Ala Asp Ser Ala Xaa Ser
410 411

(2) INFORMATION FOR SEQ ID NO:2:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1799 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCACGCGTC CGCATAAATC AGCACGCGGC CGGAGAACCC CGCAATCTCT 50

5 GCGCCCACAA AATACACCGA CGATGCCCGA TCTACTTTAA GGGCTGAAAC 100

CCACGGGCCT GAGAGACTAT AAGAGCGTTC CCTACCGCC ATG GAA 145
Met Glu

10 1

CAA CGG GGA CAG AAC GCC CCG GCC GCT TCG GGG GCC CGG 184
Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg

5 10 15

15 AAA AGG CAC GGC CCA GGA CCC AGG GAG GCG CGG GGA GCC 223
Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala
20 25

20 AGG CCT GGG CTC CGG GTC CCC AAG ACC CTT GTG CTC GTT 262
Arg Pro Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val
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25 GTC GCC GCG GTC CTG CTG TTG GTC TCA GCT GAG TCT GCT 301
Val Ala Ala Val Leu Leu Val Ser Ala Glu Ser Ala
45 50

30 CTG ATC ACC CAA CAA GAC CTA GCT CCC CAG CAG AGA GCG 340
Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln Gln Arg Ala
55 60 65

GCC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG 379
Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu
70 75 80

35

TGT CCA CCT GGA CAC CAT ATC TCA GAA GAC GGT AGA GAT 418
Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp
85 90

5 TGC ATC TCC TGC AAA TAT GGA CAG GAC TAT AGC ACT CAC 457
Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His
95 100 105

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Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys
110 115

15 GAT TCA GGT GAA GTG GAG CTA AGT CCC TGC ACC ACG ACC 535
Asp Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr
120 125 130

20 AGA AAC ACA GTG TGT CAG TGC GAA GAA GGC ACC TTC CGG 574
Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe Arg
135 140 145

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Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr
150 155

30 GGG TGT CCC AGA GGG ATG GTC AAG GTC GGT GAT TGT ACA 652
Gly Cys Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr
160 165 170

35 CCC TGG AGT GAC ATC GAA TGT GTC CAC AAA GAA TCA GGC 691
Pro Trp Ser Asp Ile Glu Cys Val His Lys Glu Ser Gly
175 180

ATC ATC ATA GGA GTC ACA GTT GCA GCC GTA GTC TTG ATT 730
Ile Ile Ile Gly Val Thr Val Ala Ala Val Val Leu Ile
35 185 190 195

GTG GCT GTG TTT GTT TGC AAG TCT TTA CTG TGG AAG AAA 769
Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys
200 205 210

5 GTC CTT CCT TAC CTG AAA GGC ATC TGC TCA GGT GGT GGT 808
Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly
215 220

10 GGG GAC CCT GAG CGT GTG GAC AGA AGC TCA CAA CGA CCT 847
Gly Asp Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro
225 230 235

15 GGG GCT GAG GAC AAT GTC CTC AAT GAG ATC GTG AGT ATC 886
Gly Ala Glu Asp Asn Val Leu Asn Glu Ile Val Ser Ile
240 245

20 TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA ATG GAA GTC 925
Leu Gln Pro Thr Gln Val Pro Glu Gln Glu Met Glu Val
250 255 260

25 CAG GAG CCA GCA GAG CCA ACA GGT GTC AAC ATG TTG TCC 964
Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser
265 270 275

30 CCC GGG GAG TCA GAG CAT CTG CTG GAA CCG GCA GAA GCT 1003
Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala
280 285

35 GAA AGG TCT CAG AGG AGG AGG CTG CTG GTT CCA GCA AAT 1042
Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala Asn
290 295 300

GAA GGT GAT CCC ACT GAG ACT CTG AGA CAG TGC TTC GAT 1081
Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp
305 310

GAC TTT GCA GAC TTG GTG CCC TTT GAC TCC TGG GAG CCG 1120
Asp Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro
315 320 325

5 CTC ATG AGG AAG TTG GGC CTC ATG GAC AAT GAG ATA AAG 1159
Leu Met Arg Lys Leu Gly Leu Met Asp Asn Glu Ile Lys
330 335 340

GTG GCT AAA GCT GAG GCA GCG GGC CAC AGG GAC ACC TTG 1198
10 Val Ala Lys Ala Glu Ala Ala Gly His Arg Asp Thr Leu
345 350

TAC ACG ATG CTG ATA AAG TGG GTC AAC AAA ACC GGG CGA 1237
Tyr Thr Met Leu Ile Lys Trp Val Asn Lys Thr Gly Arg
15 355 360 365

GAT GCC TCT GTC CAC ACC CTG CTG GAT GCC TTG GAG ACG 1276
Asp Ala Ser Val His Thr Leu Leu Asp Ala Leu Glu Thr
370 375

20 CTG GGA GAG AGA CTT GCC AAG CAG AAG ATT GAG GAC CAC 1315
Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu Asp His
380 385 390

25 TTG TTG AGC TCT GGA AAG TTC ATG TAT CTA GAA GGT AAT 1354
Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn
395 400 405

GCA GAC TCT GCC WTG TCC TAAGTGTG ATTCTCTTCA GGAAAGTGAGA 1400
30 Ala Asp Ser Ala Xaa Ser
410 411

CCTTCCCTGG TTTACCTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC 1450

35 AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC 1500

CCATCCAACA TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACTGCAC 1550
TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT 1600
5 GTCTGGATCA TTCCGTTGT GCGTACTTTG AGATTTGGTT TGGGATGTCA 1650
TTGTTTCAC AGCACTTTT TATCCTAATG TAAATGCTT ATTATTTAT 1700
10 TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAAAAG AAAAAAAAAG 1750
GGCGGCCGCG ACTCTAGAGT CGACCTGCAG AAGCTTGGCC GCCATGGCC 1799

(2) INFORMATION FOR SEQ ID NO:3:

- 15 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 70 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

20 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGAGCCGCT CATGAGGAAG TTGGGCCTCA TGGACAATGA GATAAAGGTG 50
25 GCTAAAGCTG AGGCAGCGGG 70

(2) INFORMATION FOR SEQ ID NO:4:

- 30 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

35 -79-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATCAGGGACT TTCCGCTGGG GACTTTCCG 29

5

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- 10 (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

15

AGGATGGGAA GTGTGTGATA TATCCTTGAT 30

WHAT IS CLAIMED IS:

1. A method of modulating apoptosis in mammalian cells comprising exposing mammalian cells to Apo-2 polypeptide, said Apo-2 polypeptide having at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising amino acid residues 1 to 411 of SEQ ID NO:1.
2. A method of modulating apoptosis in mammalian cells comprising exposing mammalian cells to an extracellular domain sequence of Apo-2 polypeptide comprising amino acid residues 54 to 182 of SEQ ID NO:1.

Abstract of the Disclosure

Novel polypeptides, designated Apo-2, which are capable of modulating apoptosis are provided. Compositions including Apo-2 chimeras, nucleic acid encoding Apo-2, and antibodies to Apo-2 are
5 also provided.

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1101 CCTTTGACTC CTGGGACCC CTCATGAGGA AGTTGGCCCT CATGGACAAT GAGATAAAGG TGCTTAAGC TGAGGCACCG GGCCACAGGG ACACCTTGTA
 1102 GAAAACGTAG GACCCCTGGC GAGTACTCCT TCAACCGGA GTACCTGTT CTCTATTTCC ACCGATTTCG ACTCCCGTGC CGGGTGTCC TGTGAAACAT
 322 PheAspse RTpGluPro LeuMetArgL umetaspasn Gluilelyle umetaspasn Gluilelyle umetaspasn Gluilelyle umetaspasn GlyHisARGA spthrleutyr

 1201 CACGATGCTG ATAAAGTGGG TCAAACAAAC CGGGGGAGAT GCCTGGAGAT ACACCTGTCC GGATGCCCTGT GGATGCCCTTG GAGACGGCTGG GAGAGAGACT TGCCAAGCAG
 1202 GTGCTACGAC TATTTCACCC AGTTGTTTG GCGCGCTCTA CGGAGACAGG TGTGGACCG CCTACGGAAC CTCTCGAAC ACGGTTCTGC ACGGTTCTGA ACGGTTCTGC
 355 ThrMetIeu IleLystrp alaSNlyst rGlyArgAsp AlaservalH isthrLeule uaspalaLeu GluThrLeug lygluargle ualaLysgln

 1301 AAGATTGAGG ACCACTTGT GAGCTCTGGA AAGTTCATGT ATCTAGAAGG TAATGCAGAC TCTGGCCWTGT CCTAAGTGTG ATTCTCTTCA GGAAGTGAAGA
 1302 TTCTAACTCC TGTGAAACAA CTCGAGACCT TTCAAGTACA TAGATCTCC ATTACGTCG AGACGGAAACA GGATTCAAC TRAGAGAAGT CCTTCACCTCT
 388 LysIleGluua sphisLeule userSerGly LysPheMetT yrLeuglug1 YashalaAsp ser1axqq s eFOC*

1401 CCTTCCCTGG TTACCTTT TTCTGGAAAA AGCCCAACTG GACTCAGTC AGTAGGAAAG TGCCACAAATT GTACATGAC CGGTACTGGA AGAAACTCTC
 1402 GGAAGGGACC AAATGGAAA AAGACCTTT TCGGGTTGAC CTGAGTCAG TCATCCTTC ACGGTGTAA CAGTGTACTG GCCATGACCT TCTTGTAGAG

1501 CCATCCAACA TCACCCAGTG GATGGAAACAT CCTGTAACCT TTCACTGCAC TTGGCATTAT TTTATAAGC TGAATGTGAT ATAAGGACA CTATGAAAT
 1502 GGTAGGGTGT AGTGGGTACCT CTACCTTGTAA GGACATTGAA AACGTACGTG AACCGTAATA AAAATATTG ACTTACACTA TTATCCTGT GATACTTTA

1601 GTCTGGATCA TTCCGGTTGT GCGTACTTTG AGATTTGGTT TGGATGTCAC TGTGTTTCAC AGCACTTTT TATCCTAAATG TAAATGCTTT ATTATTAT
 1602 CAGACCTAGT AAGGCAAAAC CGCATGAAAC TCTAAACCA ACCCTACAGT ACAAAAGTG TCGTAAAAA ATAGGATTAC ATTACGAAA TAAATAATAA

1701 TTGGGCTACA TTGTAAGATC CATCTACAAA AAAAAGGGGGGGG ACTCTAGAGT CGACCTGAG AAGCTGGCC GCCATGGCC
 1702 AACCCGATGT AACATTCTAG GTAGATGTTT TTTTTTTT CCGGGGGGGC GCTGGACGTC TTGAGATCTCA GTCGAAACGG CGGTACCGG

Fig. 1 (cont.)

FIG. 2 A

MEORGONAPAASGARKRHEGPGPREARGARPGLRVPKTLVLUVAVLLVSAESA
LITOQD
LAPOQORAAPQQKRSPPSEGCPGPHHISEDGRDCISQCKYQGDYSTHWNDL
LFCRLCTRCD
SGEVELSPCTTRNTVCQCEEGTFREEDSPEMCRKCRTGCPRGMVKG
DCTPWSDIEGVH
KFSSGIITIGVTVAAVLIVAFVCKSSLWKKVULPYLKGCISGGGGDPERV
DRSSQRPGAE
NVLNEIVSILQOPTQVEQEMEVQEPAEPTGVNMILSPGESEHLLPEA
ERSQRRLLVPA
NEGDPTELRQCFFDFADLVPFDSWEPLMRKLGMDNEIKVAKAAAGHR
DTLYTMLLIKW
VNKTCRDASVHTLDALETLGERLAKQIEDHLSSGKFMYLEGNADSALS

Fig. 2 B

Apo2	FADLVPEPLMVKIGI	MDNFIKVAKAEAA	-- GHRDTL
DR4	FANIVPFDSDW	DQLMRQIDLTKNEDVRACTA	-- GPGDAL
APO3/DR3	VMDAVPARRNKEFVR	TGTRAEFLAEAVEVEIGR	-- FRDQQ
TNFR1	VVENVPPLRMKEFVR	RLGSDEHIDLDRLELONGX	- CLREAO
Fas/Apop1	IRGVMTLSQVKGFVRKNGVNEAKILDEIKNDIVQDTAEQKV		

Fig. 3

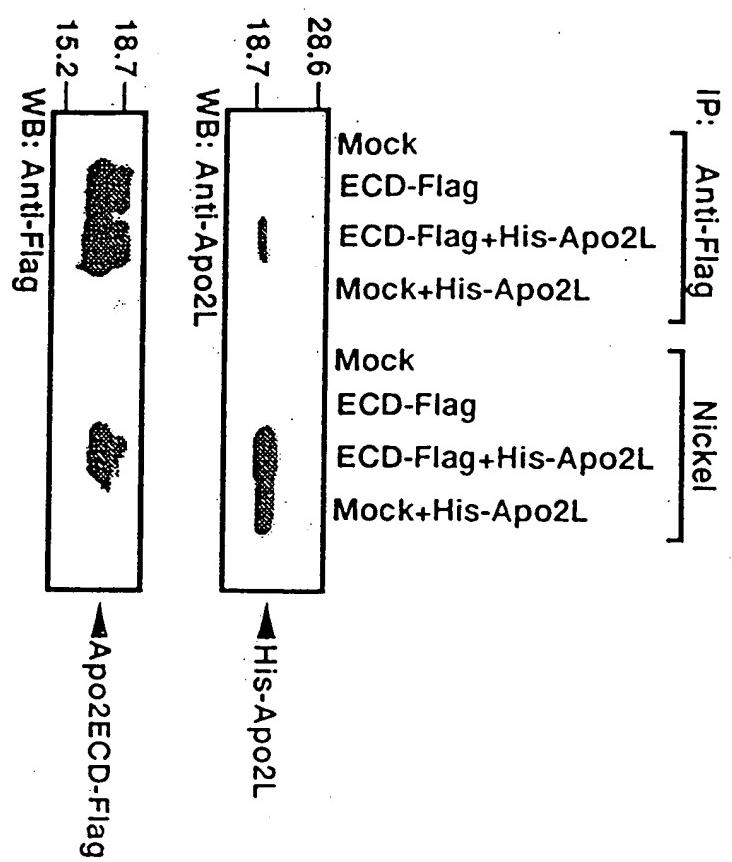


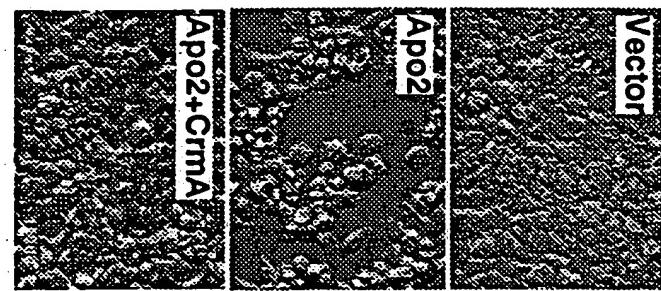
Fig. 4

4 A

4 B

4 C

4 D



Vector
Apo2
Apo2+CrmA
Apo2+DEVD
Apo2+ZVAD

4 E

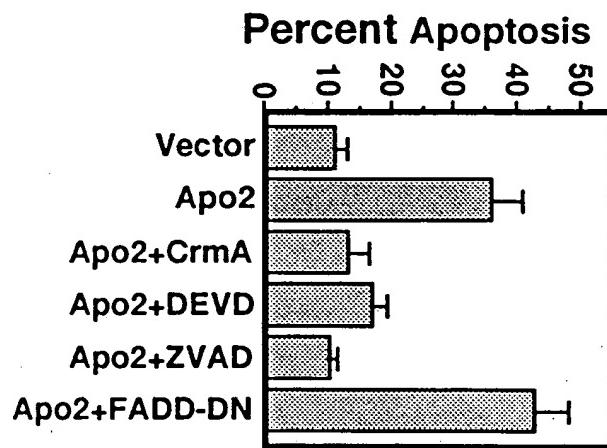
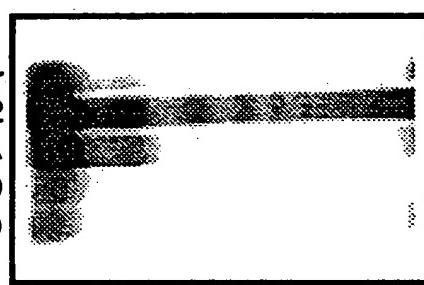
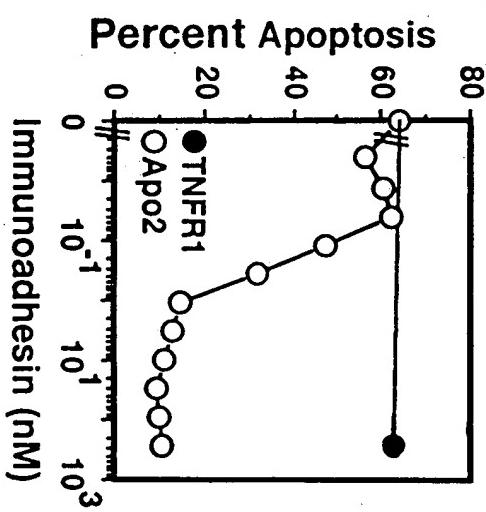
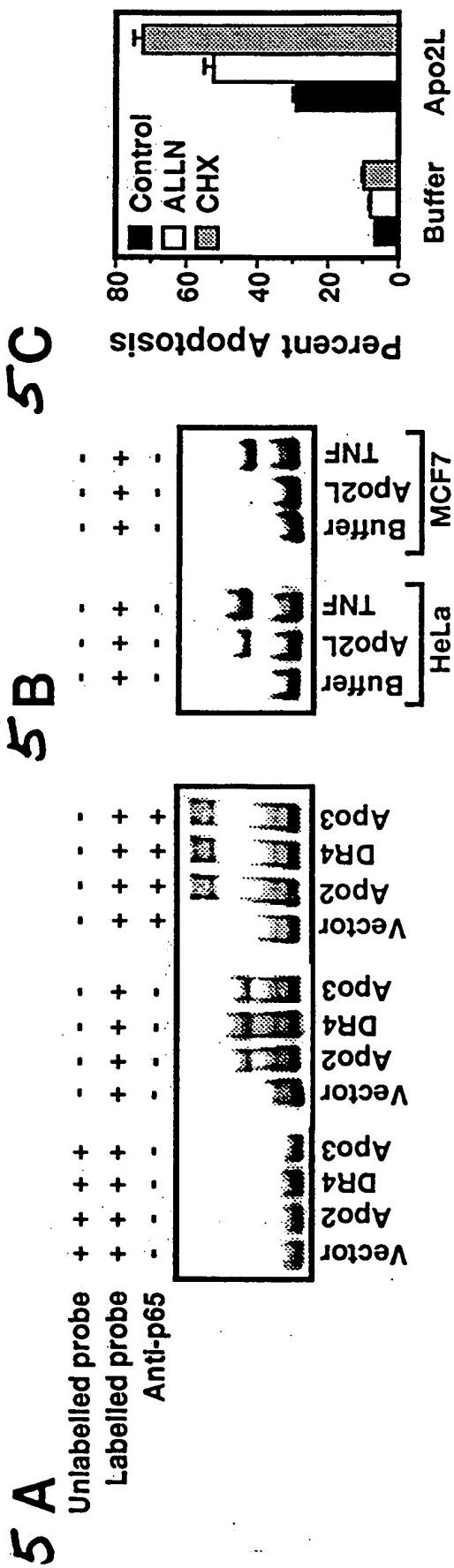


Fig. 5



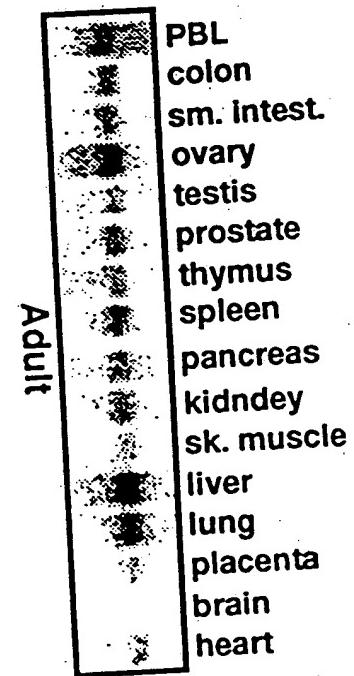
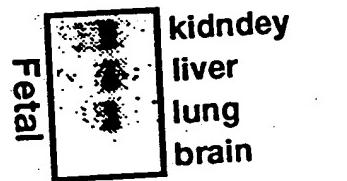


FIG. 6

60/040846

	Sub
Class	

ISSUE CLASSIFICATION
SCANNED 4

TEAM 3

PROVISIONAL
APPLICATION
NUMBER

60/040846

SERIAL NUMBER 60/040,846	FILING DATE 03/17/97	CLASS	SUBCLASS	GROUP ART UNIT	EXAMINER
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PROVISIONAL

APPLICANTS

JIAN NI, ROCKVILLE, MD; REINER L. GENTZ, SILVER SPRING, MD; GUO-HUA WU, DARNESTOWN, MD; JEFFREY SU, GAITHERSBURG, MD; CRAIG A. ROSEN, LAYTONSVILLE, MD.

CONTINUING DATA**
VERIFIED

FOREIGN/PCT APPLICATIONS***
VERIFIED

FOREIGN FILING LICENSE GRANTED 05/22/97

Foreign priority claimed 35 USC 119 conditions met	<input type="checkbox"/> yes <input type="checkbox"/> no <input checked="" type="checkbox"/> yes <input type="checkbox"/> no	AS FILED →	STATE OR COUNTRY MD	SHEETS DRAWINGS 4	TOTAL CLAIMS	INDEP. CLAIMS	FILING FEE RECEIVED \$150.00	ATTORNEY'S DOCKET NO. PF366PP
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Verified and Acknowledged
Examiner's Initials
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9410 KEY WEST AVENUE
ROCKVILLE, MD 20850

DEATH DOMAIN CONTAINING RECEPTOR 5

U.S. DEPT. OF COMM./PAT. & TM—PTO-438L (Rev.12-84)

Form PTO-1628
(Rev. 5/95)



(FACE)



60040846

APPROVED FOR LICENSE

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CONTENTS

1. Application _____ papers.

2. Request for Access

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POSITION	ID NO.	DATE
CLASSIFIER	21	5/3/87
EXAMINER	358	5-3-87
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VERIFIER	358	5-22
CORPS CORR.		
SPEC. HAND		
FILE MAINT		
DRAFTING		

PATENT APPLICATION SERIAL NO. 60/040846

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60/040846

"EXP. MAR. 17, 1997
U.S. PATENT AND TRADEMARK OFFICE
PROVISIONAL APPLICATION COVER SHEET

"Express Mail" Mailing Label Number EH7WYD2511US Date of Deposit March 17, 1997
I hereby certify that this application is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.
A. Anders Brookes
(Type or Printed Name of Person Mailing Application)
(Signature of Person Mailing Application)



This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(b)(2).

Docket No.	PF366PP	Type a plus sign (+) inside this box →	+
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■ INVENTOR (S) / APPLICANT (S)

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Su	Jeffrey		Gaithersburg, Maryland
Rosen	Craig	A	Laytonsville, Maryland

■ TITLE OF THE INVENTION (280 characters max)

Death Domain Containing Receptor 5

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Facsimile No. 301-309-8439

State	Maryland	Zip Code	20850	Country	U.S.
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■ ENCLOSED APPLICATION PARTS (check all that apply)

<input checked="" type="checkbox"/> Specification	Number of Sheets	44	<input checked="" type="checkbox"/> 19 Claims	Number of Pages 45 to 49
<input checked="" type="checkbox"/> Abstract	Number of Page(s)	50	<input checked="" type="checkbox"/> Drawings	4 sheets

■ METHOD OF PAYMENT (check one)

<input type="checkbox"/> A check or money order is enclosed to cover the Provisional filing fee	PROVISIONAL	
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 08-3428	FILING FEE AMOUNT (\$)	\$150.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

No.

Yea, the name of the U.S. Government agency and the Government contract number are:

Respectfully Submitted,

SIGNATURE

Date March 17, 1997

TYPE OR PRINTED NAME

A. Anders Brookes

REGISTRATION NO.
(if appropriate)

(Reg. No. 36,373)

Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

4

A / PAG 06/040846

-1-



Death Domain Containing Receptor 5

Field of the Invention

The present invention relates to a novel member of the tumor necrosis factor family of receptors. More specifically, isolated nucleic acid molecules are provided encoding human Death Domain Containing Receptor 5, sometimes herein "DR5". DR5 polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of DR5 activity.

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Background of the Invention

Many biological actions, for instance, response to certain stimuli and natural biological processes, are controlled by factors, such as cytokines. Many cytokines act through receptors by engaging the receptor and producing an intra-cellular response.

For example, tumor necrosis factors (TNF) alpha and beta are cytokines which act through TNF receptors to regulate numerous biological processes, including protection against infection and induction of shock and inflammatory disease. The TNF molecules belong to the "TNF-ligand" superfamily, and act together with their receptors or counter-ligands, the "TNF-receptor" superfamily. So far, nine members of the TNF ligand superfamily have been identified and ten members of the TNF-receptor superfamily have been characterized.

Among the ligands there are included TNF- α , lymphotoxin- α (LT- α , also known as TNF- β), LT- β (found in complex heterotrimer LT- $\alpha2\beta$), FasL, CD40L, CD27L, CD30L, 4-IBBL, OX40L and nerve growth factor (NGF). The superfamily of TNF receptors includes the p55TNF receptor, p75TNF receptor, TNF receptor-related protein, FAS antigen or APO-1, CD40, CD27, CD30, 4-IBB, OX40, low affinity p75 and NGF-receptor (Meager, A., *Biologics*, 22:291-295 (1994)).

Many members of the TNF-ligand superfamily are expressed by activated T-cells, implying that they are necessary for T-cell interactions with other cell types which underlie cell ontogeny and functions. (Meager, A., *supra*).

Considerable insight into the essential functions of several members of the TNF receptor family has been gained from the identification and creation of mutants that abolish the expression of these proteins. For example, naturally occurring mutations in the FAS antigen and its ligand cause lymphoproliferative disease (Watanabe-Fukunaga, R., et al., *Nature* 355:314 (1992)), perhaps reflecting a failure of programmed cell death. Mutations of the CD40 ligand cause an X-linked

immunodeficiency state characterized by high levels of immunoglobulin M and low levels of immunoglobulin G in plasma, indicating faulty T-cell-dependent B-cell activation (Allen, R.C. *et al.*, *Science* 259:990 (1993)). Targeted mutations of the low affinity nerve growth factor receptor cause a disorder characterized by faulty sensory innervation of peripheral structures (Lee, K.F. *et al.*, *Cell* 69:737 (1992)).

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TNF and LT- α are capable of binding to two TNF receptors (the 55- and 75-kd TNF receptors). A large number of biological effects elicited by TNF and LT- α , acting through their receptors, include hemorrhagic necrosis of transplanted tumors, cytotoxicity, a role in endotoxic shock, inflammation, immunoregulation, proliferation and anti-viral responses, as well as protection against the deleterious effects of ionizing radiation. TNF and LT- α are involved in the pathogenesis of a wide range of diseases, including endotoxic shock, cerebral malaria, tumors, autoimmune disease, AIDS and graft-host rejection (Beutler, B. and Von Huffel, C., *Science* 264:667-668 (1994)). Mutations in the p55 Receptor cause increased susceptibility to microbial infection.

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Moreover, an about 80 amino acid domain near the C-terminus of TNFR1 (p55) and Fas was reported as the "death domain," which is responsible for transducing signals for programmed cell death (Tartaglia *et al.*, *Cell* 74:845 (1993)).

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Apoptosis, or programmed cell death, is a physiologic process essential to the normal development and homeostasis of multicellular organisms (H. Steller, *Science* 267, 1445-1449 (1995)). Derangements of apoptosis contribute to the pathogenesis of several human diseases including cancer, neurodegenerative disorders, and acquired immune deficiency syndrome (C.B. Thompson, *Science* 267, 1456-1462 (1995)). Recently, much attention has focused on the signal transduction and biological function of two cell surface death receptors, Fas/APO-1 and TNFR-1 (J.L. Cleveland, *et al.*, *Cell* 81, 479-482 (1995); A. Fraser, *et al.*, *Cell* 85, 781-784 (1996); S. Nagata, *et al.*, *Science* 267, 1449-56 (1995)). Both are members of the TNF receptor family which also include TNFR-2, low affinity NGFR, CD40, and CD30, among others (C.A. Smith, *et al.*, *Science* 248, 1019-23 (1990); M. Tewari, *et al.*, in *Modular Texts in Molecular and Cell Biology* M. Purton; Heldin, Carl, Ed. (Chapman and Hall, London, 1995). While family members are defined by the presence of cysteine-rich repeats in their extracellular domains, Fas/APO-1 and TNFR-1 also share a region of intracellular homology, appropriately designated the "death domain", which is distantly related to the Drosophila suicide gene, reaper (P. Golstein, *et al.*, *Cell* 81, 185-6 (1995); K. White *et al.*, *Science* 264, 677-83 (1994)). This shared death domain suggests that both receptors interact with a related set of signal transducing molecules that, until recently, remained unidentified. Activation of Fas/APO-1 recruits the death

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domain-containing adapter molecule FADD/MORT1 (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M. P. Boldin, *et al.*, *J. Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO* 14, 5579-5588 (1995)), which in turn binds and presumably activates FLICE/MACH1, a member of the ICE/CED-3 family of pro-apoptotic proteases (M. Muzio *et al.*, *Cell* 85, 817-827 (1996); M.P. Boldin, *et al.*, *Cell* 85, 803-815 (1996)). While the central role of Fas/APO-1 is to trigger cell death, TNFR-1 can signal an array of diverse biological activities-many of which stem from its ability to activate NF- κ B (L.A. Tartaglia, *et al.*, *Immunol Today* 13, 151-3 (1992)). Accordingly, TNFR-1 recruits the multivalent adapter molecule TRADD, which like FADD, also contains a death domain (H. Hsu, *et al.*, *Cell* 81, 495-504 (1995); H. Hsu, *et al.*, *Cell* 84, 299-308 (1996)). Through its associations with a number of signaling molecules including FADD, TRAF2, and RIP, TRADD can signal both apoptosis and NF- κ B activation (H. Hsu, *et al.*, *Cell* 84, 299-308 (1996); H. Hsu, *et al.*, *Immunity* 4, 387-396 (1996)).

The effects of TNF family ligands and TNF family receptors are varied and influence numerous functions, both normal and abnormal, in the biological processes of the mammalian system. There is a clear need, therefore, for identification and characterization of such receptors and ligands that influence biological activity, both normally and in disease states.

Summary of the Invention

The present invention provides for isolated nucleic acid molecules comprising nucleic acid sequences encoding the amino acid sequence shown in FIG. 1 (SEQ ID NO:2) or the amino acid sequence encoding the cDNA clone deposited as ATCC Deposit No. 97920 on March 7, 1997.

The present invention also provides vectors and host cells for recombinant expression of the nucleic acid molecules described herein, as well as to methods of making such vectors and host cells and for using them for production of DRS polypeptides or peptides by recombinant techniques.

The invention further provides an isolated DRS polypeptide having an amino acid sequence encoded by a polynucleotide described herein.

The present invention also provides diagnostic assays such as quantitative and diagnostic assays for detecting levels of DRS protein. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of DRS, or soluble form thereof, compared to normal control tissue samples may be used to detect the presence of tumors.

Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including

cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes. Cellular response to TNF-family ligands include not only normal physiological responses, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis-programmed cell death is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes. Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers, autoimmune disorders, viral infections, inflammation, graft v. host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include AIDS, neurodegenerative disorders, myelodysplastic syndromes, ischemic injury, toxin-induced liver disease, septic shock, cachexia and anorexia.

15 Thus, the invention further provides a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR5 polypeptide an effective amount of an agonist capable of increasing DR5 mediated signaling. Preferably, DR5 mediated signaling is increased to treat a disease wherein decreased apoptosis is exhibited.

In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR5 polypeptide an effective amount of an antagonist capable of decreasing DR5 mediated signaling. Preferably, DR5 mediated signaling is decreased to treat a disease wherein increased apoptosis is exhibited.

Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below. Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DRS polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By the invention, a cell expressing the DRS polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

Brief Description of the Figures

FIG. 1 shows the nucleotide and deduced amino acid sequence of DRS. It is predicted that amino acids 1-51 (underlined) constitute the signal peptide, amino acids 52-184 constitute the extracellular domain, amino acids 185-208 (underlined) constitute the transmembrane domain, and amino acids 209-411 constitute the intracellular domain of which amino acids 324-391 (italicized) the death domain.

FIG. 2 shows the regions of similarity between the amino acid sequences of DRS (HLYBX88), human tumor necrosis factor receptor 1 (h TNFR1) (SEQ ID NO:3), human Fas protein (SEQ ID NO:4), and the death domain containing receptor 3 (SEQ ID NO:5). The comparison was created with the Megalign program which is contained in the DNA Star suite of programs, using the Clustal method.

FIG. 3 shows the nucleotide sequences (HAPBU13R and HSBBU76R) of two cDNA molecules which are related to the nucleotide sequence shown in Figure 1 (SEQ ID NO:1).

Detailed Description of the Preferred Embodiments

The present invention provides isolated nucleic acid molecules comprising a nucleic acid sequence encoding the DRS polypeptide whose amino acid sequence is shown in FIG. 1 (SEQ ID NO:2), or a fragment of the polypeptide. The DRS polypeptide of the present invention shares sequence homology with other known death domain containing receptors of the TNFR family including human TNFR- I, DR3 and Fas (FIG. 2). The nucleotide sequence shown in FIG. 1 (SEQ ID NO:1) was obtained by sequencing cDNA clones such as HLYBX88, which was deposited on March 7, 1997 at the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, and given Accession Number 97920. The deposited clone is contained in the pSport 1 plasmid (Life Technologies, Gaithersburg, MD).

Nucleic Acid Molecules

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide

sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

By "isolated" polypeptide or protein is intended a polypeptide or protein removed from its native environment. For example, recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for purposes of the invention as are native or recombinant polypeptides which have been substantially purified by any suitable technique such as, for example, the single-step purification method disclosed in Smith and Johnson, *Gene* 67:31-40 (1988).

Using the information provided herein, such as the nucleic acid sequence set out in FIG. 1, a nucleic acid molecule of the present invention encoding a DR5 polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as starting material. Illustrative of the invention, the genes of the present invention have also been identified in cDNA libraries of the following tissues: primary dendritic cells, endothelial tissue, spleen, chronic lymphocytic leukemia, and human thymus stromal cells.

The DR5 gene contains an open reading frame encoding a protein of about 411 amino acid residues whose initiation codon is at position 130-132 of the nucleotide sequence shown in FIG. 1 (SEQ ID NO.1), with a leader sequence of about 51 amino acid residues. Of known members of the TNF receptor family, the DR5 polypeptide of the invention shares the greatest degree of homology with human TNFR1, FAS and DR3 polypeptides shown in Fig. 2, including significant sequence homology over multiple cysteine-rich domains. The homology DR5 shows to other death domain containing receptors strongly indicates that DR5 is also a death domain containing receptor with the ability to induce apoptosis.

As indicated, the present invention also provides the mature form(s) of the DR5 protein of the present invention. According to the signal hypothesis, proteins secreted by mammalian cells have a signal or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Most mammalian cells and even

insect cells cleave secreted proteins with the same specificity. However, in some cases, cleavage of a secreted protein is not entirely uniform, which results in two or more mature species on the protein. Further, it has long been known that the cleavage specificity of a secreted protein is ultimately determined by the primary structure of the complete protein, that is, it is inherent in the amino acid sequence of the polypeptide. Therefore, the present invention provides a nucleotide sequence encoding the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clones contained in the host identified as ATCC Deposit No. 97920, and as shown in Figure 1 (SEQ ID NO:2). By the mature DR5 protein having the amino acid sequence encoded by the cDNA clones contained in the host identified as ATCC Deposit No. 97920, is meant the mature form(s) of the DR5 protein produced by expression in a mammalian cell (e.g., COS cells, as described below) of the complete open reading frame encoded by the human DNA sequence of the clone contained in the vector in the deposited host. As indicated below, the mature DR5 having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920, may or may not differ from the predicted "mature" DR5 protein shown in Figure 1 (amino acids from about 52 to about 411) depending on the accuracy of the predicted cleavage site based on computer analysis.

Methods for predicting whether a protein has a secretory leader as well as the cleavage point for that leader sequence are available. For instance, the method of McGeoch (*Virus Res.* 3:271-286 (1985)) and von Heinje (*Nucleic Acids Res.* 14:4683-4690 (1986)) can be used. The accuracy of predicting the cleavage points of known mammalian secretory proteins for each of these methods is in the range of 73-80%. von Heinje, *supra*. However, the two methods do not always produce the same predicted cleavage point(s) for a given protein.

In the present case, the predicted amino acid sequence of the complete DR5 polypeptide of the present invention was analyzed by a computer program ("PSORT"). (see K. Nakai and M. Kanehisa, *Genomics* 14:897-911 (1992)), which is an expert system for predicting the cellular location of a protein based on the amino acid sequence. As part of this computational prediction of localization, the methods of McGeoch and von Heinje are incorporated. The analysis by the PSORT program predicted the cleavage sites between amino acids 51 and 52 in Figure 1 (SEQ ID NO:2). Thereafter, the complete amino acid sequences were further analyzed by visual inspection, applying a simple form of the (-1,-3) rule of von Heinje. von Heinje, *supra*. Thus, the leader sequence for the DR5 protein is predicted to consist of amino acid residues 1-51, underlined in Figure 1 (SEQ ID NO:2), while the predicted mature DR5 protein consists of residues 52-411.

As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Isolated nucleic acid molecules of the present invention include DR5 DNA molecules comprising an open reading frame (ORF) shown in FIG. 1 (SEQ ID NO:1) and further include DNA molecules which comprise a sequence substantially different than all or part of the ORF whose initiation codon is at position 130-132 of the nucleotide sequence shown in FIG. 1 (SEQ ID NO:1) but which, due to the degeneracy of the genetic code, still encode the DR5 polypeptide or a fragment thereof. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate variants.

In another aspect, the invention provides isolated nucleic acid molecules encoding the DR5 polypeptide having an amino acid sequence encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97920 on March 7, 1997. Preferably, these nucleic acid molecules will encode the mature polypeptide encoded by the above-described deposited cDNA clone. The invention further provides an isolated nucleic acid molecule having the nucleotide sequence shown in Figure 1 (SEQ ID NO:1) or the nucleotide sequence of the DR5 cDNA contained in the above-described deposited clone, or a nucleic acid molecule having a sequence complementary to one of the above sequences. Such isolated DNA molecules and fragments thereof are useful as DNA probes for gene mapping by *in situ* hybridization of the DR5 gene in human tissue by Northern blot analysis.

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By fragments of an isolated DNA molecule having the nucleotide sequence shown in FIG. 1 (SEQ ID NO:1) are intended DNA fragments at least 20 bp, and more preferably at least 30 bp in length which are

useful as DNA probes as discussed above. Of course larger DNA fragments 50-1500 bp in length are also useful as DNA probes according to the present invention as are DNA fragments corresponding to most, if not all, of the nucleotide sequence shown in FIG. 1 (SEQ ID NO:1). By a fragment at least 20 bp in length, for example, is intended fragments which include 20 or more bases from the nucleotide sequence in FIG. 1 (SEQ ID NO:1).

Preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising the DR5 extracellular domain (amino acid residues from about 52 to about 184 in FIG. 1 (SEQ ID NO:2)); a polypeptide comprising the DR5 transmembrane domain (amino acid residues from about 185 to about 208 in FIG. 1 (SEQ ID NO:2)); a polypeptide comprising the DR5 intracellular domain (amino acid residues from about 209 to about 411 in FIG. 1 (SEQ ID NO:2)); and a polypeptide comprising the DR5 death domain (amino acid residues from about 324 to about 391 in FIG. 1 (SEQ ID NO:2)). Since the location of these domains have been predicted by computer graphics, one of ordinary skill would appreciate that the amino acid residues constituting these domains may vary slightly (e.g., by about 1 to 15 residues) depending on the criteria used to define the domain.

Preferred nucleic acid fragments of the invention encode a full-length DR5 polypeptide lacking the nucleotides encoding the amino-terminal methionine (nucleotides 130-132 in SEQ ID NO:1) as it is known that the methionine is cleaved naturally and such sequences maybe useful in genetically engineering DR5 expression vectors. Polypeptides encoded by such polynucleotides are also contemplated by the invention.

Preferred nucleic acid fragments of the present invention further include nucleic acid molecules encoding epitope-bearing portions of the DR5 protein. In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising amino acid residues from about 62 to about 110 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 119 to about 164 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 224 to about 271 in Figure 1 (SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 275 to about 370 in Figure 1 (SEQ ID NO:2). The inventors have determined that the above polypeptide fragments are antigenic regions of the DR5 protein. Methods for determining other such epitope-bearing portions of the DR5 protein are described in detail below.

In addition, the invention provides nucleic acid molecules having nucleotide sequences related to extensive portions of SEQ ID NO:1 which have been

determined from the following related cDNA clones: HAPBU13R (SEQ ID NO:6) and HSBBU76R (SEQ ID NO:7). The nucleotide sequences of HAPBU13R and HSBBU76R are shown in Figure 4.

Further, the invention includes a polynucleotide comprising any portion of at least about 30 nucleotides, preferably at least about 50 nucleotides, of SEQ ID NO:1 from residue 284 to 1,362, preferably from 284 to 681.

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the cDNA clones contained in ATCC Deposit No. 97920. By "stringent hybridization conditions" is intended overnight incubation at 42 C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65 C.

By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., the deposited cDNA or the nucleotide sequence as shown in Figure 1 (SEQ ID NO:1)).

Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3' terminal poly(A) tract of the DR5 cDNA shown in Figure 1 (SEQ ID NO:1)), or to a complementary stretch of T (or U) residues, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

As indicated, nucleic acid molecules of the present invention which encode the DR5 polypeptide may include, but are not limited to the coding sequence for the mature polypeptide, by itself; the coding sequence for the mature polypeptide and additional sequences, such as those encoding a leader or secretary sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences.

together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing - including splicing and polyadenylation signals, for example - ribosome binding and stability of mRNA; additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, for instance, the polypeptide may be fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The HA tag corresponds to an epitope derived of influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell* 37:767 (1984), for instance.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode for fragments, analogs or derivatives of the DR5 polypeptide. Variants may occur naturally, such as an allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions which may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions.

Further embodiments of the invention include isolated nucleic acid molecules that are at least 90% identical, and more preferably at least 95%, 96%, 97%, 98% or 99% identical, to (a) a nucleotide sequence encoding the full-length DR5 polypeptide having the complete amino acid sequence in Figure 1 (SEQ ID NO:2), including the predicted leader sequence; (b) nucleotide sequence encoding the full-length DR5 polypeptide having the complete amino acid sequence in Figure 1 (SEQ ID NO:2), including the predicted leader sequence but lacking the amino terminal methionine; (c) a nucleotide sequence encoding the mature DR5 polypeptide (full-length polypeptide with the leader removed) having the amino acid sequence at positions about 52 to about 411 in Figure 1 (SEQ ID NO:2); (d) a nucleotide sequence encoding the full-length DR5 polypeptide having the complete

amino acid sequence including the leader encoded by the cDNA clone contained in ATCC Deposit No. 97920; (e) a nucleotide sequence encoding the full-length DR5 polypeptide having the complete amino acid sequence including the leader but lacking the amino terminal methionine encoded by the cDNA clone contained in ATCC Deposit No. 97920; (f) a nucleotide sequence encoding the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920; (g) a nucleotide sequence that encodes the DR5 extracellular domain having the amino acid sequence at positions about 52 to about 184 of SEQ ID NO:2, or the DR5 extracellular domain encoded by the cDNA contained in ATCC Deposit No. 97920; (h) a nucleotide sequence that encodes the DR5 transmembrane domain having the amino acid sequence at positions about 185 to about 208 of SEQ ID NO:2, or the DR5 transmembrane domain encoded by the cDNA contained in ATCC Deposit No. 97920; (i) a nucleotide sequence that encodes the DR5 intracellular domain having the amino acid sequence at positions about 209 to about 411 of SEQ ID NO:2, or the DR5 intracellular domain encoded by the cDNA contained in ATCC Deposit No. 97920; (j) a nucleotide sequence that encodes the DR5 death domain having the amino acid sequence at positions about 324 to about 391 of SEQ ID NO:2, or the DR5 death domain encoded by the cDNA contained in ATCC Deposit No. 97920; and (k) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), (i), or (j) above.

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding a DR5 polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the DR5 polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in Figure 1 or to the nucleotide sequences of the deposited cDNA

clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711. Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

The present application is directed to nucleic acid molecules at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1) or to the nucleic acid sequence of the deposited cDNAs, irrespective of whether they encode a polypeptide having DR5 activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having DR5 activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having DR5 activity include, *inter alia*, (1) isolating the DR5 gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the DR5 gene, as described in Verma *et al.*, *Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York (1988); and (3) Northern Blot analysis for detecting DR5 mRNA expression in specific tissues.

Preferred, however, are nucleic acid molecules having sequences at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1) or to the nucleic acid sequence of the deposited cDNAs which do, in fact, encode a polypeptide having DR5 protein activity. By "a polypeptide having DR5 activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to an activity of the DR5 protein of the invention (either the full-length protein or, preferably, the mature protein), as measured in a particular biological assay. For example, DR5 protein activity can be measured using the cell death assays performed essentially as previously described (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO J* 14, 5579-5588 (1995); A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)) and as set forth in

Example 5, below. In MCF7 cells, plasmids encoding full-length DR5 or a candidate death domain containing receptor are co-transfected with the pLantern reporter construct encoding green fluorescent protein. Nuclei of cells transfected with DR5 will exhibit apoptotic morphology as assessed by DAPI staining. Similar to TNFR-1 and Fas/APO-1 (M. Muzio, *et al.*, *Cell* 85, 817-827 (1996); M. P. Boldin, *et al.*, *Cell* 85, 803-815 (1996); M. Tewari, *et al.*, *J Biol Chem* 270, 3255-60 (1995)), DR5-induced apoptosis is preferably blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk.

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the nucleic acid sequence of the deposited cDNA or the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1) will encode a polypeptide "having DR5 protein activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having DR5 protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J.U. *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions.

Polynucleotide assays

This invention is also related to the use of the DR5 polynucleotides to detect complementary polynucleotides such as, for example, as a diagnostic reagent. Detection of a mutated form of DR5 associated with a dysfunction will provide a diagnostic tool that can add or define a diagnosis of a disease or susceptibility to a disease which results from under-expression over-expression or altered expression of DR5 or a soluble form thereof, such as, for example, tumors or autoimmune disease.

Individuals carrying mutations in the DR5 gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy

material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR prior to analysis. (Saiki *et al.*, *Nature* 324:163-166 (1986)). RNA or cDNA may also be used in the same ways. As an example, PCR primers complementary to the nucleic acid encoding DRS can be used to identify and analyze DRS expression and mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled DRS RNA or alternatively, radiolabeled DRS antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Sequence differences between a reference gene and genes having mutations also may be revealed by direct DNA sequencing. In addition, cloned DNA segments may be employed as probes to detect specific DNA segments. The sensitivity of such methods can be greatly enhanced by appropriate use of PCR or another amplification method. For example, a sequencing primer is used with double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotide or by automatic sequencing procedures with fluorescent-tags.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels, with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, *Science* 230:1242 (1985)).

Sequence changes at specific locations also may be revealed by nuclease protection assays, such as RNase and SI protection or the chemical cleavage method (e.g., Cotton *et al.*, *Proc. Natl. Acad. Sci. USA* 85: 4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., restriction fragment length polymorphisms ("RFLP") and Southern blotting of genomic DNA.

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations also can be detected by *in situ* analysis.

Chromosome assays

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

In certain preferred embodiments in this regard, the cDNA herein disclosed is used to clone genomic DNA of a DR5 gene. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA is used for *in situ* chromosome mapping using well known techniques for this purpose.

In addition, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes.

Fluorescence *in situ* hybridization ("FISH") of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60. For a review of this technique, see Verma *et al.*, *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance in Man*, available on line through Johns Hopkins University, Welch Medical Library. The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes)).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

Vectors and Host Cells

The present invention also relates to vectors which include DNA molecules of the present invention, host cells which are genetically engineered with vectors of

the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells can be genetically engineered to incorporate nucleic acid molecules and express polypeptides of the present invention. The polynucleotides may be introduced alone or with other polynucleotides. Such other polynucleotides may be introduced independently, co-introduced or introduced joined to the polynucleotides of the invention.

In accordance with this aspect of the invention the vector may be, for example, a plasmid vector, a single or double-stranded phage vector, a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. Viral vectors may be replication competent or replication defective. In the latter case viral propagation generally will occur only in complementing host cells.

Preferred among vectors, in certain respects, are those for expression of polynucleotides and polypeptides of the present invention. Generally, such vectors comprise cis-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate trans-acting factors either are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

A great variety of expression vectors can be used to express a polypeptide of the invention. Such vectors include chromosomal, episomal and virus-derived vectors e.g., vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids, all may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides to express a polypeptide in a host may be used for expression in this regard.

The DNA sequence in the expression vector is operatively linked to appropriate expression control sequence(s), including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include the phage lambda PL promoter, the *E. coli lac*, *trp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name just a few of the well-known promoters. In general, expression constructs will contain sites for transcription, initiation and termination, and, in the transcribed region, a ribosome binding site for

translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

5 In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

10 Vectors for propagation and expression generally will include selectable markers. Such markers also may be suitable for amplification or the vectors may contain additional markers for this purpose. In this regard, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. Preferred markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing *E. coli* and other bacteria.

15 The vector containing the appropriate DNA sequence as described elsewhere herein, as well as an appropriate promoter, and other appropriate control sequences, may be introduced into an appropriate host using a variety of well known techniques suitable to expression therein of a desired polypeptide. Representative examples of appropriate hosts include bacterial cells, such as *E. coli*, Streptomyces and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila S2* and *Spodoptera Sf9* cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Hosts for a great variety of expression constructs are well known, and those of skill will be enabled by the present disclosure readily to select a host for expressing a polypeptides in accordance with this aspect of the present invention.

20 Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16A, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRITS available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG 30 available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. These vectors are listed solely by way of illustration of the many commercially available and well known vectors available to those of skill in the art.

25 Selection of appropriate vectors and promoters for expression in a host cell is a well known procedure and the requisite techniques for expression vector construction, introduction of the vector into the host and expression in the host are routine skills in the art.

The present invention also relates to host cells containing the above-described constructs discussed above. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al., Basic Methods in Molecular Biology* (1986).

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, region also may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilize proteins. For example, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobulin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery, for example, human proteins, such as, hIL-5- has been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. See, D. Bennett *et al., Journal of Molecular Recognition*, Vol. 8:52-58 (1995) and K. Johanson *et al., The Journal of Biological Chemistry*, Vol. 270, No. 16:9459-9471 (1995).

The DRS polypeptides can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography,

phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification.

Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

DR5 polynucleotides and polypeptides may be used in accordance with the present invention for a variety of applications, particularly those that make use of the chemical and biological properties of DR5. Among these are applications in treatment of tumors, resistance to parasites, bacteria and viruses, to induce proliferation of T-cells, endothelial cells and certain hematopoietic cells, to treat restenosis, graft vs. host disease, to regulate anti-viral responses and to prevent certain autoimmune diseases after stimulation of DR5 by an agonist. Additional applications relate to diagnosis and to treatment of disorders of cells, tissues and organisms. These aspects of the invention are discussed further below.

DR5 Polypeptides and Fragments

The invention further provides an isolated DR5 polypeptide having the amino acid sequence shown in FIG. 1 (SEQ ID NO:2) or a fragment thereof. It will be recognized in the art that some amino acid sequence of DR5 can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity. Such areas will usually comprise residues which make up the ligand binding site or the death domain, or which form tertiary structures which affect these domains.

Thus, the invention further includes variations of the DR5 protein which show substantial DR5 protein activity or which include regions of DR5 such as the protein fragments discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated above, guidance

concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U. *et al.*, *Science* 247:1306-1310 (1990).

Of particular interest are substitutions of charged amino acids with another charged amino acids and with neutral or negatively charged amino acids. The latter results in proteins with reduced positive charge to improve the characteristics of the DR5 protein. The prevention of aggregation is highly desirable. Aggregation of proteins not only results in a loss of activity but can also be problematic when preparing pharmaceutical formulations, because they can be immunogenic. (Pinckard *et al.*, *Clin Exp. Immunol.* 2:331-340 (1967); Robbins *et al.*, *Diabetes* 36:838-845 (1987); Cleland *et al.* *Crit. Rev. Therapeutic Drug Carrier Systems* 10:307-377 (1993)).

The replacement of amino acids can also change the selectivity of binding to cell surface receptors. Ostade *et al.*, *Nature* 361:266-268 (1993) describes certain mutations resulting in selective binding of TNF-alpha to only one of the two known types of TNF receptors. Thus, the DR5 receptor of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation.

As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 1).

TABLE 1. Conservative Amino Acid Substitutions.

Aromatic	Phenylalanine Tryptophan Tyrosine
Hydrophobic	Leucine Isoleucine Valine
Polar	Glutamine Asparagine
Basic	Arginine Lysine Histidine
Acidic	Aspartic Acid Glutamic Acid
Small	Alanine Serine Threonine Methionine Glycine

Amino acids in the DR5 protein of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as receptor binding or *in vitro*, or *in vitro* proliferative activity. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al.*, *J. Mol. Biol.* 224:899-904 (1992) and de Vos *et al.* *Science* 255:306-312 (1992)).

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. A recombinantly produced version of the DR5 polypeptide is substantially purified by the one-step method described in Smith and Johnson, *Gene* 67:31-40 (1988).

The polypeptides of the present invention also include the polypeptide encoded by the deposited cDNA including the leader, the polypeptide encoded by the deposited cDNA including the leader but excluding the N-terminal methionine, the mature polypeptide encoded by the deposited cDNA minus the leader (i.e., the mature protein), the polypeptide of Figure 1 (SEQ ID NO:2) including the leader, the polypeptide of Figure 1 (SEQ ID NO:2) minus the amino terminal methionine, the polypeptide of Figure 1 (SEQ ID NO:2) minus the leader, the extracellular domain, the transmembrane domain, the intracellular domain, the death domain, soluble polypeptides comprising all or part of the extracellular and intracellular domains but lacking the transmembrane domain as well as polypeptides which are at least 80% identical, more preferably at least 90% or 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to the polypeptide encoded by the deposited cDNA clones, to the polypeptide of Figure 1 (SEQ ID NO:2) and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a DR5 polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the DR5 polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a

number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in Figure 1 (SEQ ID NO:2) or to the amino acid sequence encoded by deposited cDNA clones can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

The present inventors have discovered that the DR5 polypeptide is a 411 residue protein exhibiting three main structural domains. First, the ligand binding domain was identified within residues from about 52 to about 184 in FIG. 1 (SEQ ID NO:2). Second, the transmembrane domain was identified within residues from about 185 to about 208 in FIG. 1 (SEQ ID NO:2). Third, the intracellular domain was identified within residues from about 209 to about 411 in FIG. 1 (SEQ ID NO:2). Importantly, the intracellular domain includes a death domain at residues from about 324 to about 391. Further preferred fragments of the polypeptide shown in FIG. 1 (SEQ ID NO:2) include the mature protein from residues about 52 to about 411 and soluble polypeptides comprising all or part of the extracellular and intracellular domains but lacking the transmembrane domain.

The invention further provides DR5 polypeptides encoded by the deposited cDNA clone including the leader and DR5 polypeptide fragments selected from the mature protein, the extracellular domain, the transmembrane domain, the intracellular domain, and the death domain.

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide described herein. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen.

On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen *et al.*, *Proc. Natl. Acad. Sci. USA* 81:3998-4002 (1983).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G., Shinnick, T. M., Green, N. and Learner, R.A. (1983) Antibodies that react with predetermined sites on proteins. *Science* 219:660-666. Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. See, for instance, Wilson *et al.*, *Cell* 37:767-778 (1984) at 777.

Antigenic epitope-bearing peptides and polypeptides of the invention preferably contain a sequence of at least seven, more preferably at least nine and most preferably between at least about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate DR5-specific antibodies include: a polypeptide comprising amino acid residues from about 62 to about 110 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 119 to about 164 in Figure 1 (SEQ ID NO:2); a polypeptide comprising amino acid residues from about 224 to about 271 in Figure 1 (SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 275 to about 370 in Figure 1 (SEQ ID NO:2). As indicated above, the inventors have determined that the above polypeptide fragments are antigenic regions of the DR5 protein.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means. Houghten, R.A., "General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen-antibody interaction at the level of individual amino acids," *Proc. Natl. Acad. Sci. USA* 82:5131-5135 (1985). This "Simultaneous Multiple Peptide

Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten *et al.* (1986).

As one of skill in the art will appreciate, DR5 polypeptides of the present invention and the epitope-bearing fragments thereof described above can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life *in vivo*. This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EPA 394,827; Traunecker *et al.*, *Nature* 331:84- 86 (1988)). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing other molecules than the monomeric DR5 protein or protein fragment alone (Fountoulakis *et al.*, *J Biochem* 270:3958-3964 (1995)).

Polypeptide assays

The present invention also relates to diagnostic assays such as quantitative and diagnostic assays for detecting levels of DR5 protein, or the soluble form thereof, in cells and tissues, including determination of normal and abnormal levels. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of DR5, or soluble form thereof, compared to normal control tissue samples may be used to detect the presence of tumors, for example. Assay techniques that can be used to determine levels of a protein, such as a DR5 protein of the present invention, or a soluble form thereof, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and ELISA assays.

Assaying DR5 protein levels in a biological sample can occur using any art-known method. Preferred for assaying DR5 protein levels in a biological sample are antibody-based techniques. For example, DR5 protein expression in tissues can be studied with classical immunohistological methods. (Jalkanen, M., *et al.*, *J. Cell. Biol.* 101:976-985 (1985); Jalkanen, M., *et al.*, *J. Cell. Biol.* 105:3087-3096 (1987)).

Other antibody-based methods useful for detecting DR5 protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA).

Suitable labels are known in the art and include enzyme labels, such as glucose oxidase, radioisotopes, such as iodine (^{125}I , ^{131}I), carbon (^{14}C), sulphur

(³⁵S), tritium (³H), indium (¹¹¹In), and technetium (^{99m}Tc), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Therapeutics

The Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes (Goeddel, D.V. *et al.*, "Tumor Necrosis Factors: Gene Structure and Biological Activities," *Symp. Quant. Biol.* 51:597-609 (1986), Cold Spring Harbor; Beutler, B., and Cerami, A., *Annu. Rev. Biochem.* 57:505-518 (1988); Old, L.J., *Sci. Am.* 258:59-75 (1988); Fiers, W., *FEBS Lett.* 285:199-224 (1991)). The TNF-family ligands induce such various cellular responses by binding to TNF-family receptors, including the DR5 of the present invention. Cells which express the DR5 polypeptide and are believed to have a potent cellular response to DR5 ligands include primary dendritic cells, endothelial tissue, spleen, chronic lymphocytic leukemia, and human thymus stromal cells. By "a cellular response to a TNF-family ligand" is intended any genotypic, phenotypic, and/or morphologic change to a cell, cell line, tissue, tissue culture or patient that is induced by a TNF-family ligand. As indicated, such cellular responses include not only normal physiological responses to TNF-family ligands, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis-programmed cell death is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes (Ameisen, J.C., *AXDS* 8:1197-1213 (1994); Krammer, P.H. *et al.*, *Curr. Opin. Immunol.* 6:279-289 (1994)).

Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, such as breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as systemic lupus erythematosus and immune-related glomerulonephritis rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), information graft v. host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration); myclodysplastic syndromes (such as aplastic anemia), ischemic injury (such as that caused by

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myocardial infarction, stroke and reperfusion injury), toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia.

Thus, in one aspect, the present invention is directed to a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR5 polypeptide an effective amount of DR5 ligand, analog or an agonist capable of increasing DR5 mediated signaling. Preferably, DR5 mediated signaling is increased to treat a disease wherein decreased apoptosis or decreased cytokine and adhesion molecule expression is exhibited. An agonist can include soluble forms of DR5 and monoclonal antibodies directed against the DR5 polypeptide.

In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR5 polypeptide an effective amount of an antagonist capable of decreasing DR5 mediated signaling. Preferably, DR5 mediated signaling is decreased to treat a disease wherein increased apoptosis or NFkB expression is exhibited. An antagonist can include soluble forms of DR5 and monoclonal antibodies directed against the DR5 polypeptide.

By "agonist" is intended naturally occurring and synthetic compounds capable of enhancing or potentiating apoptosis. By "antagonist" is intended naturally occurring and synthetic compounds capable of inhibiting apoptosis. Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below.

One such screening procedure involves the use of melanophores which are transfected to express the receptor of the present invention. Such a screening technique is described in PCT WO 92/01810, published February 6, 1992. Such an assay may be employed, for example, for screening for a compound which inhibits (or enhances) activation of the receptor polypeptide of the present invention by contacting the melanophore cells which encode the receptor with both a TNF-family ligand and the candidate antagonist (or agonist). Inhibition or enhancement of the signal generated by the ligand indicates that the compound is an antagonist or agonist of the ligand/receptor signaling pathway.

Other screening techniques include the use of cells which express the receptor (for example, transfected CHO cells) in a system which measures extracellular pH changes caused by receptor activation, for example, as described in *Science* 246:181-196 (October 1989). For example, compounds may be contacted with a cell which expresses the receptor polypeptide of the present invention and a

second messenger response, e.g., signal transduction or pH changes, may be measured to determine whether the potential compound activates or inhibits the receptor.

Another such screening technique involves introducing RNA encoding the receptor into *Xenopus* oocytes to transiently express the receptor. The receptor oocytes may then be contacted with the receptor ligand and a compound to be screened, followed by detection of inhibition or activation of a calcium signal in the case of screening for compounds which are thought to inhibit activation of the receptor.

Another screening technique involves expressing in cells a construct wherein the receptor is linked to a phospholipase C or D. Such cells include endothelial cells, smooth muscle cells, embryonic kidney cells, etc. The screening may be accomplished as hereinabove described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase signal.

Another method involves screening for compounds which inhibit activation of the receptor polypeptide of the present invention antagonists by determining inhibition of binding of labeled ligand to cells which have the receptor on the surface thereof. Such a method involves transfecting a eukaryotic cell with DNA encoding the receptor such that the cell expresses the receptor on its surface and contacting the cell with a compound in the presence of a labeled form of a known ligand. The ligand can be labeled, e.g., by radioactivity. The amount of labeled ligand bound to the receptors is measured, e.g., by measuring radioactivity of the receptors. If the compound binds to the receptor as determined by a reduction of labeled ligand which binds to the receptors, the binding of labeled ligand to the receptor is inhibited.

Further screening assays for agonist and antagonist of the present invention are described in Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267(7):4304-4307(1992).

Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DR5 polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the

ligand/receptor signaling pathway. By "assaying a cellular response" is intended qualitatively or quantitatively measuring a cellular response to a candidate compound and/or a TNF-family ligand (e.g., determining or estimating an increase or decrease in T cell proliferation or tritiated thymidine labeling). By the invention, a cell expressing the DR5 polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

Agonist according to the present invention include naturally occurring and synthetic compounds such as, for example, TNF family ligand peptide fragments, transforming growth factor , neurotransmitters (such as glutamate, dopamine, *N*-methyl-D-aspartate), tumor suppressors (p53), cytolytic T cells and antimetabolites. Preferred agonist include chemotherapeutic drugs such as, for example, cisplatin, doxorubicin, bleomycin, cytosine arabinoside, nitrogen mustard, methotrexate and vincristine. Others include ethanol and -amyloid peptide. (*Science* 267:1457-1458 (1995)). Further preferred agonist include polyclonal and monoclonal antibodies raised against the DR5 polypeptide, or a fragment thereof. Such agonist antibodies raised against a TNF-family receptor are disclosed in Tartaglia, L.A., *et al.*, *Proc. Natl. Acad. Sci. USA* 88:9292-9296 (1991); and Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267 (7):4304-4307 (1992) See, also, PCT Application WO 94/09137.

Antagonist according to the present invention include naturally occurring and synthetic compounds such as, for example, the CD40 ligand, neutral amino acids, zinc, estrogen, androgens, viral genes (such as Adenovirus *EIB*, Baculovirus *p35* and *IAP*, Cowpox virus *crmA*, Epstein-Barr virus *BHRFI*, *LMP-1*, African swine fever virus *LMW5-HL*, and Herpesvirus *yl 34.5*), calpain inhibitors, cysteine protease inhibitors, and tumor promoters (such as PMA, Phenobarbital, and -Hexachlorocyclohexane).

Other potential antagonists include antisense molecules. Antisense technology can be used to control gene expression through antisense DNA or RNA or through triple-helix formation. Antisense techniques are discussed, for example, in Okano, *J. Neurochem.* 56:560 (1991); *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance Lee *et al.*, *Nucleic Acids Research* 6:3073 (1979); Cooney *et al.*, *Science* 241:456 (1988); and Dervan *et al.*, *Science* 251:1360 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.

For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA

oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into receptor polypeptide. The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the receptor.

Further antagonist according to the present invention include soluble forms of DR5, i.e., DR5 fragments that include the ligand binding domain from the extracellular region of the full length receptor. Such soluble forms of the receptor, which may be naturally occurring or synthetic, antagonize DR5 mediated signaling by competing with the cell surface DR5 for binding to TNF-family ligands. Thus, soluble forms of the receptor that include the ligand binding domain are novel cytokines capable of inhibiting apoptosis induced by TNF-family ligands. These are preferably expressed as dimers or trimers, since these have been shown to be superior to monomeric forms of soluble receptor as antagonists, e.g., IgGFc-TNF receptor family fusions. Other such cytokines are known in the art and include Fas B (a soluble form of the mouse Fas receptor) that acts physiologically to limit apoptosis induced by Fas ligand (Hughes, D.P. and Crispe, I.N., *J. Exp. Med.* 182:1395-1401 (1995)).

The term "antibody" (Ab) or "monoclonal antibody" (mAb) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and F(ab')₂ fragments) which are capable of binding an antigen. Fab and F(ab')₂ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al.*, *J. Nucl. Med.* 24:316-325 (1983)).

Antibodies according to the present invention may be prepared by any of a variety of methods using DR5 immunogens of the present invention. As indicated, such DR5 immunogens include the full length DR5 polypeptide (which may or may not include the leader sequence) and DR5 polypeptide fragments such as the ligand binding domain, the transmembrane domain, the intracellular domain and the death domain.

Proteins and other compounds which bind the DR5 domains are also candidate agonist and antagonist according to the present invention. Such binding compounds can be "captured" using the yeast two-hybrid system (Fields and Song, *Nature* 340:245-246 (1989)). A modified version of the yeast two-hybrid system has been described by Roger Brent and his colleagues (Gyuris, J. *et al.*, *Cell* 75:791-803 (1993); Zervos, A.S. *et al.*, *Cell* 72:223-232 (1993)). Preferably, the yeast two-hybrid system is used according to the present invention to capture

compounds which bind to either the DRS ligand binding domain or to the DRS intracellular domain. Such compounds are good candidate agonist and antagonist of the present invention.

By a "TNF-family ligand" is intended naturally occurring, recombinant, and synthetic ligands that are capable of binding to a member of the TNF receptor family and inducing the ligand/receptor signaling pathway. Members of the TNF ligand family include, but are not limited to, DRS ligands, TRAIL, TNF- α , lymphdtoksin- α (LT- α , also known as TNF- β), LT- β (found in complex heterotrimer LT- α 2- β), FasL, CD40, CD27, CD30, 4-IBB, OX40 and nerve growth factor (NGF).

Representative therapeutic applications of the present invention are discussed in more detail below. The state of immunodeficiency that defines AIDS is secondary to a decrease in the number and function of CD4 $^{+}$ T-lymphocytes. Recent reports estimate the daily loss of CD4 $^{+}$ T cells to be between 3.5×10^7 and 2×10^9 cells (Wei X., et al., *Nature* 373:117-122 (1995)). One cause of CD4 $^{+}$ T cell depletion in the setting of HIV infection is believed to be HIV-induced apoptosis. Indeed, HIV-induced apoptotic cell death has been demonstrated not only *in vitro* but also, more importantly, in infected individuals (Ameisen, J.C., *AIDS* 8:1197-1213 (1994); Finkel, T.H., and Banda, N.K., *Curr. Opin. Immunol.* 6:605-615(1995); Muro-Cacho, C.A. et al., *J. Immunol.* 154:5555-5566 (1995)). Furthermore, apoptosis and CD4 $^{+}$ T-lymphocyte depletion is tightly correlated in different animal models of AIDS (Brunner, T., et al., *Nature* 373:441-444 (1995); Gougeon, M.L., et al., *AIDS Res. Hum. Retroviruses* 9:553-563 (1993)) and, apoptosis is not observed in those animal models in which viral replication does not result in AIDS (Gougeon, M.L. et al., *AIDS Res. Hum. Retroviruses* 9:553-563 (1993)). Further data indicates that uninfected but primed or activated T lymphocytes from HIV-infected individuals undergo apoptosis after encountering the TNF-family ligand FasL. Using monocytic cell lines that result in death following HIV infection, it has been demonstrated that infection of U937 cells with HIV results in the *de novo* expression of FasL and that FasL mediates HIV-induced apoptosis (Badley, A.D. et al., *J. Virol.* 70:199-206 (1996)). Further the TNF-family ligand was detectable in uninfected macrophages and its expression was upregulated following HIV infection resulting in selective killing of uninfected CD4 T-lymphocytes (Badley, A.D. et al., *J. Virol.* 70:199-206 (1996)). Thus, by the invention, a method for treating HIV $^{+}$ individuals is provided which involves administering an antagonist of the present invention to reduce selective killing of CD4 T-lymphocytes. Modes of administration and dosages are discussed in detail below.

In rejection of an allograft, the immune system of the recipient animal has not previously been primed to respond because the immune system for the most part is only primed by environmental antigens. Tissues from other members of the same species have not been presented in the same way that, for example, viruses and bacteria have been presented. In the case of allograft rejection, immunosuppressive regimens are designed to prevent the immune system from reaching the effector stage. However, the immune profile of xenograft rejection may resemble disease recurrence more than allograft rejection. In the case of disease recurrence, the immune system has already been activated, as evidenced by destruction of the native islet cells. Therefore, in disease recurrence the immune system is already at the effector stage. Agonist of the present invention are able to suppress the immune response to both allografts and xenografts because lymphocytes activated and differentiated into effector cells will express the DR5 polypeptide, and thereby are susceptible to compounds which enhance apoptosis. Thus, the present invention further provides a method for creating immune privileged tissues. Antagonist of the invention can further be used in the treatment of Inflammatory Bowel-Disease.

DR5 antagonists may be useful for treating inflammatory diseases, such as rheumatoid arthritis, osteoarthritis, psoriasis, septicemia, and inflammatory bowel disease.

In addition, due to lymphoblast expression of DR5, soluble DR5, agonist or antagonist mABs may be used to treat this form of cancer. Further, soluble DR5 or neutralizing mABs may be used to treat various chronic and acute forms of inflammation such as rheumatoid arthritis, osteoarthritis, psoriasis, septicemia, and inflammatory bowel disease.

Modes of Administration

The agonist or antagonists described herein can be administered *in vitro*, *ex vivo*, or *in vivo* to cells which express the receptor of the present invention. By administration of an "effective amount" of an agonist or antagonist is intended an amount of the compound that is sufficient to enhance or inhibit a cellular response to a TNF-family ligand and include polypeptides. In particular, by administration of an "effective amount" of an agonist or antagonists is intended an amount effective to enhance or inhibit DR5 mediated apoptosis. Of course, where apoptosis is to be enhanced, an agonist according to the present invention can be co-administered with a TNF-family ligand. One of ordinary skill will appreciate that effective amounts of an agonist or antagonist can be determined empirically and may be employed in pure form or in pharmaceutically acceptable salt, ester or prodrug form. The agonist or

antagonist may be administered in compositions in combination with one or more pharmaceutically acceptable excipients.

It will be understood that, when administered to a human patient, the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgement. The specific therapeutically effective dose level for any particular patient will depend upon factors well known in the medical arts.

As a general proposition, the total pharmaceutically effective amount of DRS polypeptide administered parenterally per dose will be in the range of about 1 µg/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given continuously, the DRS agonists or antagonists is typically administered at a dose rate of about 1 µg/kg/hour to about 50 µg/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed.

Dosaging may also be arranged in a patient specific manner to provide a predetermined concentration of an agonist or antagonist in the blood, as determined by the RIA technique. Thus patient dosaging may be adjusted to achieve regular on-going trough blood levels, as measured by RIA, on the order of from 50 to 1000 ng/ml, preferably 150 to 500 ng/ml.

Pharmaceutical compositions are provided comprising an agonist or antagonist and a pharmaceutically acceptable carrier or excipient, which may be administered orally, rectally, parenterally, intracistemally, intravaginally, intraperitoneally, topically (as by powders, ointments, drops or transdermal patch), buccally, or as an oral or nasal spray. Importantly, by co-administering an agonist and a TNF-family ligand, clinical side effects can be reduced by using lower doses of both the ligand and the agonist. It will be understood that the agonist can be "co-administered" either before, after, or simultaneously with the TNF-family ligand, depending on the exigencies of a particular therapeutic application. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Pharmaceutical compositions of the present invention for parenteral injection can comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions,

dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use.

In addition to soluble DRS polypeptides, DRS polypeptide containing the transmembrane region can also be used when appropriately solubilized by including detergents, such as CHAPS or NP-40, with buffer.

Example 1

Expression and Purification in E. coli

The DNA sequence encoding the mature DRS protein in the deposited cDNA clone (ATCC No. 97920) is amplified using PCR oligonucleotide primers specific to the amino terminal sequences of the DRS protein and to vector sequences 3' to the gene. Additional nucleotides containing restriction sites to facilitate cloning are added to the 5' and 3' sequences respectively.

The following primers are used for expression of DRS extracellular domain in *E. coli*: The 5' primer has the sequence 5'-CGCCCATGGAGTCTGCTCTGATCAC -3' (SEQ ID NO:8) and contains the underlined NcoI site; and the 3' primer has the sequence 5'-CGCAAGCTTTAGCCTGATTCTTGTGGAC -3' (SEQ ID NO:9) and contains the underlined HindIII site.

The restriction sites are convenient to restriction enzyme sites in the bacterial expression vector pQE60, which are used for bacterial expression in these examples. (Qiagen, Inc. 9259 Eton Avenue, Chatsworth, CA, 91311). pQE60 encodes ampicillin antibiotic resistance ("Amp") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, and a ribosome binding site ("RBS").

The amplified DRS DNA and the vector pQE60 both are digested with NcoI and HindIII and the digested DNAs are then ligated together. Insertion of the DRS protein DNA into the restricted pQE60 vector places the DRS protein coding region downstream of and operably linked to the vector's IPTG-inducible promoter and in-frame with an initiating AUG appropriately positioned for translation of DRS protein.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures. Such procedures are described in Sambrook *et al.*, Molecular Cloning: a Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses lac repressor and confers kanamycin resistance ("Kan"), is used in carrying out the illustrative example described herein.

This strain, which is only one of many that are suitable for expressing DRS protein, is available commercially from Qiagen.

Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 µg/ml) and kanamycin (25 µg/ml).

The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:100 to 1:250. The cells are grown to an optical density at 600nm ("OD₆₀₀") of between 0.4 and 0.6. Isopropyl-B-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from *lac* repressor sensitive promoters, by inactivating the *lacI* repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation and disrupted, by standard methods. Inclusion bodies are purified from the disrupted cells using routine collection techniques, and protein is solubilized from the inclusion bodies into 8M urea. The 8M urea solution containing the solubilized protein is passed over a PD-10 column in 2X phosphate-buffered saline ("PBS"), thereby removing the urea, exchanging the buffer and refolding the protein. The protein is purified by a further step of chromatography to remove endotoxin. Then, it is sterile filtered. The sterile filtered protein preparation is stored in 2X PBS at a concentration of 95 µg/ml.

Example 2

Expression in Mammalian Cells

Most of the vectors used for the transient expression of a given gene sequence in mammalian cells carry the SV40 origin of replication. This allows the replication of the vector to high copy numbers in cells (e.g. COS cells) which express the T antigen required for the initiation of viral DNA synthesis. Any other mammalian cell line can also be utilized for this purpose.

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g. RSV, HTLV, HIVI and the early promoter of the cytomegalovirus (CMV).

However, also cellular signals can be used (e.g. human actin, promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC67109). Mammalian host cells that could be used include, human Hela, 283, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1 African green monkey cells, quail QC1-3 cells, mouse L cells and Chinese hamster ovary cells such as

Alternatively, a gene of interest can be expressed in stable cell lines that contain the gene integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) is a useful marker to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Using this marker, the mammalian cells are grown in increasing amounts of methotrexate for selection and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) cells are often used for the production of proteins.

The expression vectors pCl and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen *et al.*, *Molecular and Cellular Biology* 438:44701 (March 1985)), plus a fragment of the CMV-enhancer (Boshart *et al.*, *Cell* 41:521-530 (1985)). Multiple cloning sites, e.g. with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

Cloning and Expression in CHO Cells

The vector pC4 is used for the expression of DR5 polypeptide. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt; F. W., Kellems, R. M., Bertino, J. R., and Schimke, R. T., 1978, *J. Biol. Chem.*

253:1357-1370, Hamlin, J. L. and Ma, C. 1990, *Biochem. et Biophys. Acta*,
1097:107-143, Page, M. J. and Sydenham, M. A. 1991, *Biotechnology* 9:64-68).
Cells grown in increasing concentrations of MTX develop resistance to the drug by
overproducing the target enzyme, DHFR, as a result of amplification of the DHFR
5 gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and
over-expressed. It is known in the art that this approach may be used to develop
cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently,
when the methotrexate is withdrawn, cell lines are obtained which contain the
amplified gene integrated into one or more chromosome(s) of the host cell.

10 Plasmid pC4 contains for expressing the gene of interest the strong
promoter of the long terminal repeat (LTR) of the Rouse Sarcoma Virus (Cullen, et
al., *Molecular and Cellular Biology*, March 1985:438-447) plus a fragment isolated
from the enhancer of the immediate early gene of human cytomegalovirus (CMV)
(Boshart et al., *Cell* 41:521-530 (1985)). Downstream of the promoter are the
15 following single restriction enzyme cleavage sites that allow the integration of the
genes: BamHI, Xba I, and Asp718. Behind these cloning sites the plasmid
contains the 3' intron and polyadenylation site of the rat preproinsulin gene. Other
high efficiency promoters can also be used for the expression, e.g., the human β -
actin promoter, the SV40 early or late promoters or the long terminal repeats from
other retroviruses, e.g., HIV and HTLV. Clontech's Tet-Off and Tet-On gene
expression systems and similar systems can be used to express the DR5 polypeptide
20 in a regulated way in mammalian cells (Gossen, M., & Bujard, H. 1992, *Proc.
Natl. Acad. Sci. USA* 89:5547-5551). For the polyadenylation of the mRNA other
signals, e.g., from the human growth hormone or globin genes can be used as well.
Stable cell lines carrying a gene of interest integrated into the chromosomes can also
25 be selected upon co-transfection with a selectable marker such as gpt, G418 or
hygromycin. It is advantageous to use more than one selectable marker in the
beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with the restriction enzyme BamHI and then
30 dephosphorylated using calf intestinal phosphates by procedures known in the art.
The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the complete polypeptide is amplified using
PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the
desired portion of the gene. The 5' primer containing the underlined BamHI site, a
35 Kozak sequence, and an AUG start codon, has the following sequence:
5' CGCGGATCCGCCATCATGGAAACAACGGGGACAGAAC 3' (SEQ ID
NO:10). The 3' primer, containing the underlined Asp718 site, has the following

sequence: 5' CGCGGTACCTTAGGACATGGCAGAGTC 3' (SEQ ID NO:11).

The amplified fragment is digested with the endonuclease BamHI and Asp718 and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or 5 XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene are used for transfection. Five μ g of the expression plasmid pC4 is cotransfected with 0.5 μ g of the plasmid pSVneo using lipofectin (Felgner et al., *supra*). The plasmid pSV2-neo 10 contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded 15 in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 μ M, 2 μ M, 5 μ M, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a 20 concentration of 100 - 200 μ M. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

Cloning and Expression in COS Cells

The expression plasmid, pDR5HA, is made by cloning a portion of the cDNA encoding the soluble extracellular domain of the DR5 protein into the expression vector pcDNAI/Amp or pcDNAIII (which can be obtained from Invitrogen, Inc.).

The expression vector pcDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cells; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron; (5) several codons encoding a hemagglutinin fragment (i.e., an "HA" tag to facilitate purification) followed by a termination codon and polyadenylation signal arranged so that a cDNA can be conveniently placed under expression control of the CMV promoter and operably 30 35

linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson et al., *Cell* 37: 767 (1984). The fusion of the HA tag to the target protein allows easy detection and recovery of the recombinant protein with an antibody that recognizes the HA epitope. pcDNAIII contains, in addition, the selectable neomycin marker.

A DNA fragment encoding the extracellular domain of the DR5 polypeptide is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the CMV promoter. The plasmid construction strategy is as follows. The DR5 cDNA of the deposited clone is amplified using primers that contain convenient restriction sites, much as described above for construction of vectors for expression of DR5 in *E. coli*. Suitable primers include the following, which are used in this example. The 5' primer, containing the underlined BamHI site has the following sequence: 5'
10 CGCGGGATCCGCCATCATGGAACACGGGACAGAAC 3' (SEQ ID NO:10). The 3' primer, containing the underlined Asp718 restriction sequence has the following sequence: 5' CGCGGTACCTTAGCCTGATTCTTGAC 3' (SEQ ID NO:12).

The PCR amplified DNA fragment and the vector, pcDNAI/Amp, are digested with BamHI and Asp718 and then ligated. The ligation mixture is transformed into *E. coli* strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037), and the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis or other means for the presence of the fragment encoding the extracellular domain of the DR5 polypeptide

For expression of recombinant DR5, COS cells are transfected with an expression vector, as described above, using DEAE-DEXTRAN, as described, for instance, in Sambrook et al., *Molecular Cloning: a Laboratory Manual*, Cold Spring Laboratory Press, Cold Spring Harbor, New York (1989). Cells are incubated under conditions for expression of DR5 by the vector.

Expression of the DR5-HA fusion protein is detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow et al., *Antibodies: A Laboratory Manual*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988). To this end, two days after transfection, the cells are labeled by incubation in media containing ³⁵S-cysteine for 8 hours. The

cells and the media are collected, and the cells are washed and lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson et al. cited above. Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then are analyzed by SDS-PAGE and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

The primers sets used for expression in this example are compatible with pC4 used for CHO expression in this example, pcDNA1/Amp for COS expression in this example, and pA2 used for baculovirus expression in the following example. Thus, for example, the complete DRS encoding fragment amplified for CHO expression could also be ligated into pcDNA1/Amp for COS expression or pA2 for baculovirus expression.

Example 3

Cloning and expression of the soluble extracellular domain of DRS in a baculovirus expression system

The cDNA sequence encoding the soluble extracellular domain of DRS protein in the deposited clone (ATCC No. 97920) is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer for DRS has the sequence 5' CGCGGATCCGCCATCATGGAACAAACGGGGACAGAAC 3' (SEQ ID NO:10) containing the underlined BamHI restriction enzyme site. Inserted into an expression vector, as described below, the 5' end of the amplified fragment encoding DRS provides an efficient cleavage signal peptide. An efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol.* 196:947-950 (1987) is appropriately located in the vector portion of the construct.

The 3' primer for DRS has the sequence 5' CGCGGTACCTTAGCCTGATTCTTGAC 3' (SEQ ID NO:12) containing the underlined Asp718 restriction followed by nucleotides complementary to the DRS nucleotide sequence set out in FIG. 1, followed by the stop codon.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.) The fragment then is digested with BamHI and Asp718 and again is purified on a 1% agarose gel.

The vector pA2 is used to express the DRS protein in the baculovirus expression system, using standard methods, such as those described in Summers et

al., *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedron promoter of the Autograph californica nuclear polyhedrosis virus (ACMNPV) followed by convenient restriction sites. For an easy selection of recombinant virus the beta-galactosidase gene from *E. coli* is inserted in the same orientation as the polyhedron promoter and is followed by the polyadenylation signal of the polyhedron gene. The polyhedron sequences are flanked at both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that express the cloned polynucleotide.

Many other baculovirus vectors could be used in place of pA2, such as pAc373, pVL941 and pAcIM1 provided, as those of skill readily will appreciate, that construction provides appropriately located signals for transcription, translation, trafficking and the like, such as an in-frame AUG and a signal peptide, as required. Such vectors are described in Luckow *et al.*, *Virology* 170:31-39, among others.

The plasmid is digested with the restriction enzyme Bam HI and Asp718 and then is dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.).

Fragment and the dephosphorylated plasmid are ligated together with T4 DNA ligase. *E. coli* HB101 cells are transformed with ligation mix and spread on culture plates. Bacteria are identified that contain the plasmid with the human DRS gene by digesting DNA from individual colonies using BamHI and Asp718 and then analyzing the digestion product by gel electrophoresis. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pBac DRS.

5 µg of the plasmid pBac DRS is co-transfected with 1.0 µg of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.), using the lipofection method described by Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7413-7417 (1987). 1 µg of BaculoGold™ virus DNA and 5 µg of the plasmid pBac DRS are mixed in a sterile well of a microliter plate containing 50 µl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added

solution. The plate is then incubated for 5 hours at 27 C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27 C for four days.

5 After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, cited above. An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

10 Four days after serial dilution, the virus is added to the cells. After appropriate incubation, blue stained plaques are picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses is then resuspended in an Eppendorf tube containing 200 μ l of Grace's medium. The agar is removed by a brief centrifugation and the supernatant containing the recombinant baculovirus is used to infect SF9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4 C. A clone containing properly inserted DR5 is identified by DNA analysis including restriction mapping and sequencing. This is designated herein as V- DR5.

15 SF9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus V- DR5 at a multiplicity of infection ("MOI") of about 2 (about 1 to about 3). Six hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Gaithersburg). 42 hours later, 5 gCi of 35 S-methionine and 5 μ Ci 35 S cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then they are harvested by centrifugation, lysed and the labeled proteins are visualized by SDS-PAGE and autoradiography.

30 *Example 4*

Tissue distribution of DR5 gene expression

35 Northern blot analysis is carried out to examine DR5 gene (ATCC No. 97920) expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide sequence of the DR5 protein (SEQ ID NO:1) is labeled with 32 P using the rediprimeTM DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe is purified using a CHROMA

SPIN-100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to examine various human tissues for DRS mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) are obtained from Clontech and are examined with labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70 C overnight, and films developed according to standard procedures.

Example 5

DRS Induced Apoptosis

Overexpression of Fas/APO-1 and TNFR-1 in mammalian cells mimics receptor activation (M. Muzio, *et al.*, *Cell* 85, 817-827 (1996); M. P. Boldin, *et al.*, *Cell* 85, 803-815 (1996)). Thus, this system is utilized to study the functional role of DR5. Transient expression of DR5 in MCF7 human breast carcinoma cells and 293 human embryonic kidney cells is investigated for induction of rapid apoptosis.

Experimental Design

Cell death assays are performed essentially as previously described (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO J* 14, 5579-5588 (1995); A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)). Briefly, MCF-7 human breast carcinoma clonal cell lines stably transfected with either vector alone or a CrmA expression construct (M. Tewari, *et al.*, *J Biol Chem* 270, 3255-60 (1995)), are transiently transfected with pCMV-DR5-galactosidase (or pCMV-DR5-galactosidase (lacking the death domain)) in the presence of a ten-fold excess of pcDNA3 expression constructs encoding the indicated proteins using lipofectamine (GIBCO-BRL). 293 cells are likewise transfected using the CaPO₄ method. The ICE family inhibitor z-VAD-fmk (Enzyme Systems Products, Dublin, CA) is added to the cells at a concentration of 10μM, 5 hrs after transfection. 32 hours following transfection, cells are fixed and stained with X-Gal as previously described (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO J* 14, 5579-5588 (1995)).

Results

The affected cells will display morphological alterations typical of cells undergoing apoptosis, becoming rounded, condensed and detaching from the dish.

Similar to TNFR-1 and Fas/APO-1 (M. Muzio, et al., *Cell* 85, 817-827 (1996); M. P. Boldin, et al., *Cell* 85, 803-815 (1996); M. Tewari, et al., *J Biol Chem* 270, 3255-60 (1995)), DR5-induced apoptosis is preferably blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk.

5

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosures of all patents, patent applications, and publications referred to herein are hereby incorporated by reference.

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WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:
- (a) a nucleotide sequence encoding the full-length DR5 polypeptide having the complete amino acid sequence in Figure 1 (SEQ ID NO:2), including the predicted leader sequence;
- (b) a nucleotide sequence encoding the full-length DR5 polypeptide having the complete amino acid sequence in Figure 1 (SEQ ID NO:2), including the predicted leader sequence but lacking the amino terminal methionine;
- (c) a nucleotide sequence encoding the mature DR5 polypeptide (full-length polypeptide with the leader removed) having the amino acid sequence at positions about 52 to about 411 in Figure 1 (SEQ ID NO:2);
- (d) a nucleotide sequence encoding the full-length DR5 polypeptide having the complete amino acid sequence including the leader encoded by the cDNA clone contained in ATCC Deposit No. 97920;
- (e) a nucleotide sequence encoding the full-length DR5 polypeptide having the complete amino acid sequence including the leader but lacking the amino terminal methionine encoded by the cDNA clone contained in ATCC Deposit No. 97920;
- (f) a nucleotide sequence encoding the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;
- (g) a nucleotide sequence that encodes the DR5 extracellular domain having the amino acid sequence at positions about 52 to about 184 of SEQ ID NO:2, or the DR5 extracellular domain encoded by the cDNA contained in ATCC Deposit No. 97920;
- (h) a nucleotide sequence that encodes the DR5 transmembrane domain having the amino acid sequence at positions about 185 to about 208 of SEQ ID NO:2, or the DR5 transmembrane domain encoded by the cDNA contained in ATCC Deposit No. 97920;
- (i) a nucleotide sequence that encodes the DR5 intracellular domain having the amino acid sequence at positions about 209 to about 411 of SEQ ID NO:2, or the DR5 intracellular domain encoded by the cDNA contained in ATCC Deposit No. 97920;
- (j) a nucleotide sequence that encodes the DR5 death domain having the amino acid sequence at positions about 324 to about 391 of SEQ ID NO:2, or

the DR5 death domain encoded by the cDNA contained in ATCC Deposit No. 97920; and

(k) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), (i), or (j) above.

5

2. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence in Figure 1 (SEQ ID NO:1).

10

3. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figure 1 (SEQ ID NO:1) encoding the DR5 polypeptide having the amino acid sequence in positions 2 - 411 of SEQ ID NO:2.

15

4. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence in Figure 1 (SEQ ID NO:1) encoding the extracellular domain of the DR5 polypeptide having the amino-acid sequence from about 52 to about 184 in SEQ ID NO:2.

20

5. The nucleic acid molecule of claim 1 wherein said polynucleotide has the complete nucleotide sequence of the cDNA clone contained in ATCC Deposit No. 97920.

25

6. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the DR5 polypeptide having the complete amino acid sequence excepting the N-terminal methionine encoded by the cDNA clone contained in ATCC Deposit No. 97920.

30

7. The nucleic acid molecule of claim 1 wherein said polynucleotide has the nucleotide sequence encoding the extracellular domain of the DR5 polypeptide having the amino acid sequence of the extracellular domain encoded by the cDNA clone contained in ATCC Deposit No. 97920.

35

8. An isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having a nucleotide sequence identical to a nucleotide sequence in (a), (b), (c), (d), (e), (f), (g), (h), (i), (j) or (k) of claim 1 wherein said polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues.

9. An isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of a DRS polypeptide having an amino acid sequence in (a), (b), (c), (d), (e), (f), (g), (h), (i) or (j) of claim 1.

10. The isolated nucleic acid molecule of claim 10, which encodes an epitope-bearing portion of a DRS polypeptide wherein the amino acid sequence of said portion is selected from the group consisting of: a polypeptide comprising amino acid residues from about 62 to about 110 of SEQ ID NO:2, a polypeptide comprising amino acid residues from about 119 to about 164 of SEQ ID NO:2, a polypeptide comprising amino acid residues from about 224 to about 271 of SEQ ID NO:2, and a polypeptide comprising amino acid residues from about 275 to about 370 of SEQ ID NO:2.

15. A method for making a recombinant vector comprising inserting an isolated nucleic acid molecule of claim 1 into a vector.

20. A recombinant vector produced by the method of claim 11.

25. A method of making a recombinant host cell comprising introducing the recombinant vector of claim 12 into a host cell.

30. A recombinant host cell produced by the method of claim 13.

35. A recombinant method for producing a DRS polypeptide, comprising culturing the recombinant host cell of claim 14 under conditions such that said polypeptide is expressed and recovering said polypeptide.

40. An isolated DRS polypeptide comprising an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

(a) the amino acid sequence of the full-length DRS polypeptide having the complete amino acid sequence in Figure 1 (SEQ ID NO:2), including the predicted leader sequence;

(b) the amino acid sequence of the full-length DRS polypeptide having the complete amino acid sequence in Figure 1 (SEQ ID NO:2), including the predicted leader sequence but lacking the amino terminal methionine;

- (c) the amino acid sequence of the mature DRS polypeptide (full-length polypeptide with the leader removed) having the amino acid sequence at positions about 52 to about 411 in Figure 1 (SEQ ID NO:2);
- 5 (d) the amino acid sequence of the full-length DRS polypeptide having the complete amino acid sequence including the leader encoded by the cDNA clone contained in ATCC Deposit No. 97920;
- (e) the amino acid sequence of the full-length DRS polypeptide having the complete amino acid sequence including the leader but lacking the amino terminal methionine encoded by the cDNA clone contained in ATCC Deposit No. 97920;
- 10 (f) the amino acid sequence of the mature DRS polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;
- (g) the amino acid sequence of the DRS extracellular domain having the amino acid sequence at positions about 52 to about 184 of SEQ ID NO:2, or as encoded by the cDNA contained in ATCC Deposit No. 97920;
- 15 (h) the amino acid sequence of the DRS transmembrane domain having the amino acid sequence at positions about 185 to about 208 of SEQ ID NO:2, or as encoded by the cDNA contained in ATCC Deposit No. 97920;
- (i) the amino acid sequence of the DRS intracellular domain having the amino acid sequence at positions about 209 to about 411 of SEQ ID NO:2, or as encoded by the cDNA contained in ATCC Deposit No. 97920; and
- 20 (j) the amino acid sequence of the DRS death domain having the amino acid sequence at positions about 324 to about 391 of SEQ ID NO:2, or as encoded by the cDNA contained in ATCC Deposit No. 97920.
- 25 17. An isolated polypeptide comprising an epitope-bearing portion of the DRS protein, wherein said portion is selected from the group consisting of: a polypeptide comprising amino acid residues from about 62 to about 110 of SEQ ID NO:2, a polypeptide comprising amino acid residues from about 119 to about 164 of SEQ ID NO:2, a polypeptide comprising amino acid residues from about 224 to about 271 of SEQ ID NO:2, and a polypeptide comprising amino acid residues from about 275 to about 370 of SEQ ID NO:2.
- 30 18. An isolated antibody that binds specifically to a DRS polypeptide of claim 16.

19. An isolated nucleic acid molecule comprising a polynucleotide having a sequence at least 95% identical to a sequence selected from the group consisting of:

- (a) the nucleotide sequence of clone HAPBU13R (SEQ ID NO:6);
- (b) the nucleotide sequence of clone HSBBU76R (SEQ ID NO:7);
- (c) the nucleotide sequence of a portion of the sequence shown in Figure 1 (SEQ ID NO:1) wherein said portion comprises at least 50 contiguous nucleotides from nucleotide 284 to 1,362; and
- (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b) or (c) above.

Abstract

The present invention relates to novel Death Domain Containing Receptor-5 (DR5) proteins which are members of the tumor necrosis factor (TNF) receptor family. In particular, isolated nucleic acid molecules are provided encoding the human DR5 proteins. DR5 polypeptides are also provided as are vectors, host cells and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of DR5 activity.

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Figure 1

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Figure 1 (continued)

GTTCCAGCAAATGAAGGTGATCCCCTGAGACTCTGAGACAGTGCTTCGATGACTTTGCA
V P A N E G D P T E T L R Q C F D D F A
1090 1110 1130
GACTTGGTGCCTTGTACTCCTGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAAT
D L V P F D S W E P L M R K L G L M D N
1150 1170 1190
GAGATAAAAGGTGGCTAAAGCTGAGGCAGCGGCCACAGGGACACCTTGACACGATGCTG
S I K V A K A S A A G H R D T L Y T M L
1210 1230 1250
ATAAAAGTGGGTCAACAAAAACGGGGAGATGCCCTGTCCACACCCCTGCTGGATGCCCTG
I K W V N K T G R D A S V H T L L D A L
1270 1290 1310
GAGACGCTGGAGAGAGACTTGCCAAAGCAGAAGATTGAGGACCACTTGTTGAGCTCTGGA
E T L G E R L A K Q R I E D H L L S S G
1330 1350 1370
AAGTTCATGTATCTAGAAGGTAAATGCAGACTCTGCCATGTCCTAACGTGTGATTCTCTCA
K F M Y L E G N A D S A M S *
1390 1410 1430
GGAAGTGAGACCTTCCCTGGTTACCTTTCTGGAAAAAGCCCAACTGGACTCCAGTC
1450 1470 1490
AGTAGGAAAGTGCCACAATTGTACATGACGGTACTGGAAGAAACTCTCCCATCCAACA
1510 1530 1550
TCACCCAGTGGATGAAACATCCTGTAACCTTCACTGCACTTGGCATTATTTTATAAGC
1570 1590
TGAATGTGATAATAAGGACACTATGGAAAAAAAAAAAAA

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Figure 2

1	- - - - -	I W T L P E - - -	h Fas protein
1	- - - - -	T V P D E L P - - -	h TNFR I Protein
1	- - - - -	G C A A V A A	DR3 protein
1	Q G Q N A P A A S Q A R K R H Q P C P R E A R G A R P G - - - V P K T - - -	HLYBX88XXprotein	
13	T S V V R - S S K - V N A Q V T D I N S K E - - - L L E K T V T T V E T Q N L - - -	h Fas protein	
14	- - - - - L L V - I Y P S - V I G L V P H - - - D E K - D S V - P Q - - - K Y I H - - -	h TNFR I Protein	
14	A - L - - - - - A R Q - - - - - T S P - - C D A - - D F - H - - -	DR3 protein	
41	V A - - - - - L V - - - S S A L I T Q Q D - A P Q Q - A A P Q Q K R S S P S - - -	HLYBX88XXprotein	
53	H H D - Q - - - - - H K P - - - - - E R K A R E - - - V N - - - D E P I - - - V P - - - Q E K E P T - - -	h Fas protein	
52	P Q M N S I - - - T K - H K - T - - - Y N D - P G - - - Q D T - - - R E - E S S S T - - -	h TNFR I Protein	
41	R K I D L - - - - - L R G - - - - - K A P E - - - E C O N S T I L V - - - P O D T - L E - - -	DR3 protein	
81	- - - - - - - - - - - H I S E - - - - - G R A I S - K Y - Q D - S - - -	HLYBX88XXprotein	
93	D K A - - - S S K - R - - - R L - - - I G H - L E V I - - - R - Q N - K - - - - - N - - -	h Fas protein	
92	S - - - L R - H - - - S S K - R K E M - - - I S - - - V D - - - - - - - R K N - - -	h TNFR I Protein	
81	W - - - H H - - - A - - - Q A - - - Q A S - - - A B - - - S A V A - - - R - - - - -	DR3 protein	
105	T H W N D L L F - - - T R - - - S E - - - P - - - T - - - N - - - M - - - Q - - - E E - - -	HLYBX88XXprotein	
133	P -	h Fas protein	
131	Q Y - - - H Y W S E R N L F -	h TNFR I Protein	
121	W - V C - - - - - Q V S - - - V B - - - Y Q P - - - D - - - A L - - - R H T - - - L S - - -	DR3 protein	
143	T -	HLYBX88XXprotein	
149	- -	h Fas protein	
161	K Q N T - C - - - H A - - - P L R E H -	h TNFR I Protein	
158	R D T D C - - - - - L -	DR3 protein	
163	G N V K - - - D -	HLYBX88XXprotein	
168	- -	h Fas protein	
201	W V K G T - D - - - O T T - - - L P L - - - I P -	h TNFR I Protein	
197	W R Q -	DR3 protein	
189	- - - - - V T V A A - - - V V I V A V -	HLYBX88XXprotein	
190	V - R - - - E V Q K - - - R - H E K - - - N - O - S H - S - - - - - - - - - - - - - - - - - - -	h Fas protein	
240	- - - S - - - L Y S I V G - - - S T P - - - K - - - E - - - L - - - T T K - - - A P N P - - - F S P T - - - G - - -	h TNFR I Protein	
229	- - - P H - - - P L - - - V A - - - B A G H - - - A L T P P P A - - - B L S - - - D S A R T L - - - A - - - D - - -	DR3 protein	
221	- -	HLYBX88XXprotein	
213	- -	h Fas protein	
279	F T P T L G F S P V - - - S S T F - - - S S S T Y - - - P G D - - - - - - - - - - - - - - - - - -	h TNFR I Protein	
267	S S - - - K I C T - - - L V G N S W - - - P G Y - - - E Q Q E A - - - - - - - - - - - - - - - - -	DR3 protein	
255	V P - - - Q E H E - - - E -	HLYBX88XXprotein	
213	- -	h Fas protein	
318	Y Q Q A D - - - I L T A - - - A S D P I P N P - - - Q K W - - - S - - - R K - - - Q S L D T D D - - - A - - -	h TNFR I Protein	
305	S R A L Q G - - - A A -	DR3 protein	
283	- -	HLYBX88XXprotein	
241	- - - S Q -	h Fas protein	
358	- - - A - - - V N - - - E - - - P L - - - H - - - P - - - R - - - E - - - R - - - L - - - L Q - - - C L - - -	h TNFR I Protein	
335	- -	DR3 protein	
312	C T D - - - P A L - - - L - - - F D S - - - P L M - - - M - - - M - - - K - - - A K A - - - A G H - - -	HLYBX88XXprotein	
272	B - E K V O L - - - R N - - - H - - - L - - - K - - - K - - - Y D - - - I K D - - - K K A N - - - C T L A - - - K - - -	h Fas protein	
398	B A D - - - S - - - A T - - - R R - - - P R - - - A T - - - B L - - - G R V - - - R D - - - D - - - L - - - L - - -	h TNFR I Protein	
173	E - - - E - - - K R - - - Q Q P -	DR3 protein	
351	T L - - - T - - - I K - - - V N K - - - D - - - S V H - - - L D - - - - - - - T L - - - E R - - - L A K Q K - - -	HLYBX88XXprotein	
311	Q T I I - - - K D - - - I T S D S E N - - - F R M E I Q S - V -	h Fas protein	
438	Q E A -	h TNFR I Protein	
410	- -	DR3 protein	
390	Q D H - - - S S G K F M Y L B Q - - - A D S A M S -	HLYBX88XXprotein	

Decoration 'Decoration 01': Shade (with solid black) residues that match the Consensus exactly.

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Figure 3

HAPBU13R

1 AATTCCGGCAC AGCTCTTCAG GAAGTCAGAC CTTCCCTGGT TTACCTTTTT
51 TCTGGAAAAA GCCCAACTGG GACTCCAGTC AGTAGGAAAG TGCCACAATT
101 GTCACATGAC CGGTACTGGA AGAAAACTCTC CCATCCAACA TCACCCAGTG
151 GNATGGGAAC ACTGATGAAC TTTTCACTGC ACTTGGCATT ATTTTTGTNA
201 AGCTGAATGT GATAATAAGG GCACTGATGG AAATGTCCTGG ATCATTCCGG
251 TTGTGCGTAC TTTGAGATTT GNGTTTGGGG ATGTNCATTG TGTTTGACAG
301 CACTTTTTTN ATCCCTTAATG TNAAATGCNT NATTGATTG TGANTTGGGG
351 GTNAACATTTG GTNAAGGNNTN CCCNTNTGAC ACAGTAGNTG GTNCCCCGACT
401 TANAATNGNN GAANANGATG NATNANGAAC CTCCCCCTGG GTGGGGGGGT
451 NNCGGGGAG TNNAANGNNG NCTCCCCAGG TTTGGNGTNG CAATNGNGGA
501 ANNNNTGG

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HSBBU76R

1 TTTTTTTTGT AGATGGATCT TACAATGTAG CCCAAATAAA TAAATAAAGC
51 ATTTACATTA GGATAAAAAA GTGCTGTGAA AACAAATGACA TCCCAAACCA
101 AATCTCAAAG TACGCACAAA CGGAATGATC CAGACATTTC CATAGNGTCC
151 TTATTATCAC ATTCAAGCTTA TAAAANTAAAT GCCAAGTGCA GTGAAAAGTT
201 ACAGGATGTT CCATCCACTG GGTGGATT

Serial No.	507054,021	Issue Date	07/29/97
Class	Subclass	SCANNED	

SERIAL NUMBER 507054,021	FILING DATE 07/29/97	CLASS	SUBCLASS	GROUP ART UNIT	EXAMINER
PROVISIONAL					

JIAN NI, ROCKVILLE, MD; REINER L. GENTZ, SILVER SPRING, MD; YAO-LIANG DARNESTOWN, MD; JEFFREY Y. SU, GAITHERSBURG, MD; CRAIG A. RODEN, LAYTONSVILLE, MD.

CONTINUING DATA***
VERIFIED

FOREIGN/PCT APPLICATIONS***
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* FOREIGN FILING LICENSE GRANTED 11/06/97

Foreign priority claimed 35 USC 119 conditions met	<input type="checkbox"/> yes <input type="checkbox"/> no	<input type="checkbox"/> yes <input type="checkbox"/> no	AS FILED →	STATE OR COUNTRY MD	SHEETS DRAWGS. 7	TOTAL CLAIMS	INDEP. CLAIMS	FILING FEE RECEIVED \$150.00	ATTORNEY'S DOCKET NO. PF366PP2
Verified and Acknowledged	Examiner's Initials								
ROBERT H BENSON A ANDERS BROOKES HUMAN GENOME SCIENCES INC 9410 KEY WEST AVENUE ROCKVILLE MD 20850									

DEATH DOMAIN CONTAINING RECEPTOR 5

TITLE

U.S. DEPT. OF COMM./PAT. & TM—PTO-436L (Rev.12-94)



PATENT APPLICATION



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"Express Mail" Mailing Label Number EHT791302691US Date of Deposit July 29, 1997
 I hereby certify that this application is being deposited with the United States Postal Service
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Rachel Ann Hilborn
 (Type or Printed Name of Person Mailing Application)
Rachel A. Hilborn
 (Signature of Person Mailing Application)

A/PHLV

**U.S. PATENT AND TRADEMARK OFFICE
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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(b)(2).

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◊ INVENTOR (S) / APPLICANT (S)			
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Death Domain Containing Receptor 5			
◊ CORRESPONDENCE ADDRESS			
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		Country	U.S.
◊ ENCLOSED APPLICATION PARTS (check all that apply)			
<input checked="" type="checkbox"/> Specification No. of Pages: 51 (1-51)		<input checked="" type="checkbox"/> Abstract No. of Pages: 1 (Page: 58)	
<input checked="" type="checkbox"/> 24 Claims No. of Pages 6 (52-57)		<input checked="" type="checkbox"/> Figures No. of Sheets: 7 (Figures 1-6)	
◊ METHOD OF PAYMENT (check one)			
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<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees and credit Deposit Account Number 08-3425			FILING FEE AMOUNT (\$) \$150.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

No.

Yes, the name of the U.S. Government agency and the Government contract number are:

Respectfully Submitted,

SIGNATURE

Date July 29, 1997

Type or PRINTED NAME

A. Anders Brookes

REGISTRATION NO.
(if appropriate)

Reg. No. 36,373

Additional inventors are being named on separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

Death Domain Containing Receptor 5

Background of the Invention

5 *Field of the Invention*

The present invention relates to a novel member of the tumor necrosis factor family of receptors. More specifically, isolated nucleic acid molecules are provided encoding human Death Domain Containing Receptor 5, or simply "DR5." DR5 polypeptides are also provided, as are vectors, host cells, and recombinant methods for producing the same. The invention further relates to screening methods for identifying agonists and antagonists of DR5 activity.

10 *Related Art*

Many biological actions, for instance, response to certain stimuli and natural biological processes, are controlled by factors, such as cytokines. Many cytokines act through receptors by engaging the receptor and producing an intracellular response.

15 For example, tumor necrosis factors (TNF) alpha and beta are cytokines, which act through TNF receptors to regulate numerous biological processes, including protection against infection and induction of shock and inflammatory disease. The TNF molecules belong to the "TNF-ligand" superfamily, and act together with their receptors or counter-ligands, the "TNF-receptor" superfamily. So far, nine members of the TNF ligand superfamily have been identified and ten members of the TNF-receptor superfamily have been characterized.

20 Among the ligands, there are included TNF- α , lymphotoxin- α (LT- α , also known as TNF- β), LT- β (found in complex heterotrimer LT- α - β - γ), FasL, CD40L, CD27L, CD30L, 4-IBBL, OX40L and nerve growth factor (NGF). The superfamily of TNF receptors includes the p55TNF receptor, p75TNF receptor, TNF receptor-related protein, FAS antigen or APO-1, CD40, CD27, CD30, 4-IBB, OX40, low affinity p75 and NGF-receptor (Meager, A., *Biologicals*, 22:291-295 (1994)).

25 Many members of the TNF-ligand superfamily are expressed by activated T-cells, implying that they are necessary for T-cell interactions with other cell types which underlie cell ontogeny and functions. (Meager, A., *supra*).

30 Considerable insight into the essential functions of several members of the TNF receptor family has been gained from the identification and creation of

mutants that abolish the expression of these proteins. For example, naturally occurring mutations in the FAS antigen and its ligand cause lymphoproliferative disease (Watanabe-Fukunaga, R., et al., *Nature* 356:314 (1992)), perhaps reflecting a failure of programmed cell death. Mutations of the CD40 ligand cause an X-linked immunodeficiency state characterized by high levels of immunoglobulin M and low levels of immunoglobulin G in plasma, indicating faulty T-cell-dependent B-cell activation (Allen, R.C. et al., *Science* 259:990 (1993)). Targeted mutations of the low affinity nerve growth factor receptor cause a disorder characterized by faulty sensory innervation of peripheral structures (Lee, K.F. et al., *Cell* 69:737 (1992)).

TNF and LT- α are capable of binding to two TNF receptors (the 55- and 75-kd TNF receptors). A large number of biological effects elicited by TNF and LT- α , acting through their receptors, include hemorrhagic necrosis of transplanted tumors, cytotoxicity, a role in endotoxic shock, inflammation, immunoregulation, proliferation and anti-viral responses, as well as protection against the deleterious effects of ionizing radiation. TNF and LT- α are involved in the pathogenesis of a wide range of diseases, including endotoxic shock, cerebral malaria, tumors, autoimmune disease, AIDS and graft-host rejection (Beutler, B. and Von Huffel, C., *Science* 264:667-668 (1994)). Mutations in the p55 Receptor cause increased susceptibility to microbial infection.

Moreover, an about 80 amino acid domain near the C-terminus of TNFR1 (p55) and Fas was reported as the "death domain," which is responsible for transducing signals for programmed cell death (*Tartaglia et al., Cell* 74:843 (1993)).

Apoptosis, or programmed cell death, is a physiologic process essential for the normal development and homeostasis of multicellular organisms (H. Steller, *Science* 267, 1443-1449 (1995)). Derangements of apoptosis contribute to the pathogenesis of several human diseases including cancer, neurodegenerative disorders, and acquired immune deficiency syndrome (C.B. Thompson, *Science* 267, 1456-1462 (1995)). Recently, much attention has focused on the signal transduction and biological function of two cell surface death receptors, Fas/APO-1 and TNFR-1 (J.L. Cleveland et al., *Cell* 81, 479-482 (1995); A. Fraser, et al., *Cell* 85, 781-784 (1996); S. Nagata et al., *Science* 267, 1449-56 (1995)). Both are members of the TNF receptor family which also include TNFR-2, low affinity NGFR, CD40, and CD30, among others (C.A. Smith et al., *Science* 248, 1019-23 (1990); M. Tewari et al., in *Modular Texts in Molecular and Cell Biology* M. Purton, Heldin, Carl, Ed. (Chapman and Hall, London, 1995)). While family members are defined by the presence

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of cysteine-rich repeats in their extracellular domains, Fas/APO-1 and TNFR-1 also share a region of intracellular homology, appropriately designated the "death domain", which is distantly related to the Drosophila suicide gene, reaper (P. Golstein, *et al.*, *Cell* 81, 185-186 (1995); K. White *et al.*, *Science* 264, 677-83 (1994)). This shared death domain suggests that both receptors interact with a related set of signal transducing molecules that, until recently, remained unidentified. Activation of Fas/APO-1 recruits the death domain-containing adapter molecule FADD/MORT1 (A.M. Chinnaiyan *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin *et al.*, *J. Biol. Chem.* 270, 7795-8 (1995); F.C. Kischkel *et al.*, *EMBO J.* 14, 5579-5588 (1995)), which in turn binds and presumably activates FLICE/MACH1, a member of the ICE/CED-3 family of pro-apoptotic proteases (M. Muzio *et al.*, *Cell* 85, 817-827 (1996); M.P. Boldin *et al.*, *Cell* 85, 803-815 (1996)). While the central role of Fas/APO-1 is to trigger cell death, TNFR-1 can signal an array of diverse biological activities-many of which stem from its ability to activate NF-kB (L.A. Tartaglia *et al.*, *Immuno Today* 13, 151-3 (1992)). Accordingly, TNFR-1 recruits the multivalent adapter molecule TRADD, which like FADD, also contains a death domain (H. Hsu *et al.*, *Cell* 81, 495-504 (1995); H. Hsu, *et al.*, *Cell* 84, 299-308 (1996)). Through its associations with a number of signaling molecules including FADD, TRAF2, and RIP, TRADD can signal both apoptosis and NF-kB activation (H. Hsu *et al.*, *Cell* 84, 299-308 (1996); H. Hsu, *et al.*, *Immunity* 4, 387-396 (1996)).

Recently, a new apoptosis -inducing TNF ligand has been discovered. S.R. Wiley *et al.*, *Immunity* 3,673-682 (1995) named the molecule - "TNF-related apoptosis-inducing ligand" or simply "TRAIL." The molecule was also called "Apo-2 ligand" or "Apo-2L." R.M. Pitt *et al.*, *J. Biol. Chem.* 271,12687-12690 (1996). This molecule was also disclosed in co-pending U.S. provisional application no. 60/013,405. For convenience, the molecule will be referred to herein as TRAIL.

Unlike FAS ligand, whose transcripts appear to be largely restricted to stimulated T-cells, significant levels of TRAIL are detected in many human tissues (e.g., spleen, lung, prostate, thymus, ovary, small intestine, colon, peripheral blood lymphocytes, placenta, kidney), and is constitutively transcribed by some cell lines. It has been shown that TRAIL acts independently from the Fas ligand (Wiley *et al.*, *supra*). It has also been shown that TRAIL activates apoptosis rapidly, within a time frame that is similar to death signalling by Fas/Apo-1L, but much faster than TNF-induced apoptosis. S.A. Marsters *et al.*, *Current Biology* 6, 750-752 (1996). The inability of TRAIL to bind

TNFR-1, Fas, or the recently identified DR3, suggests that TRAIL may interact with a unique receptor(s).

Several unique receptors for TRAIL have already been identified. In co-pending U.S. provisional application no. 60/035,722, DR4, a novel death domain containing receptor for TRAIL, was disclosed. See, Pan *et al.*, *Science* 276,111-113 (April 1997). The TR5 receptor, the subject of co-pending U.S. provisional patent application 60/035,496, has now been shown to bind TRAIL. In co-pending U.S. provisional patent application 60/xxxxxx, it was predicted that the TR10 receptor would also bind TRAIL, owing to sequence homology with DR4.

The effects of TNF family ligands and TNF family receptors are varied and influence numerous functions, both normal and abnormal, in the biological processes of the mammalian system. There is a clear need, therefore, for identification and characterization of such receptors and ligands that influence biological activity, both normally and in disease states. In particular, there is a need to isolate and characterize additional novel receptors that bind TRAIL.

Summary of the Invention

The present invention provides for isolated nucleic acid molecules comprising nucleic acid sequences encoding the amino acid sequence shown in FIG. 1 (SEQ ID NO:2) or the amino acid sequence encoded by the cDNA clone deposited as ATCC Deposit No. 97920 on March 7, 1997.

The present invention also provides recombinant vectors, which include the isolated nucleic acid molecules of the invention, and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells and for using them for production of DRS polypeptides or peptides by recombinant techniques.

The invention further provides an isolated DRS polypeptide having an amino acid sequence encoded by a polynucleotide described herein.

The present invention also provides diagnostic assays such as quantitative and diagnostic assays for detecting levels of DRS protein. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of DRS, or soluble form thereof, compared to normal control tissue samples may be used to detect the presence of tumors.

Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes. Cellular response to TNF-family

ligands include not only normal physiological responses, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis - programmed cell death - is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes. Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers, autoimmune disorders, viral infections, inflammation, graft v. host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include AIDS, neurodegenerative disorders, myelodysplastic syndromes, ischemic injury, toxin-induced liver disease, septic shock, cachexia and anorexia.

Thus, the invention further provides a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR5 polypeptide an effective amount of an agonist capable of increasing DR5 mediated signaling. Preferably, DR5 mediated signaling is increased to treat a disease wherein decreased apoptosis is exhibited.

In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR5 polypeptide an effective amount of an antagonist capable of decreasing DR5 mediated signaling. Preferably, DR5 mediated signaling is decreased to treat a disease wherein increased apoptosis is exhibited.

Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below. Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DR5 polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By the invention, a cell expressing the DR5 polypeptide can be

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contacted with either an endogenous or exogenously administered TNF-family ligand.

Brief Description of the Figures

FIG. 1 shows the nucleotide (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of DR5. It is predicted that amino acids 1-51 (underlined) constitute the signal peptide (amino acid residues from about -51 to about -1 in SEQ ID NO:2); amino acids 52-184 constitute the extracellular domain (amino acid residues from about 1 to about 133 in SEQ ID NO:2); amino acids 185-208 (underlined) constitute the transmembrane domain (amino acid residues from about 134 to about 157 in SEQ ID NO:2); and amino acids 209-411 constitute the intracellular domain (amino acid residues from about 158 to about 360 in SEQ ID NO:2), of which amino acids 324-391 (italicized) constitute the death domain (amino acid residues from about 273 to about 340 in SEQ ID NO:2).

FIG. 2 shows the regions of similarity between the amino acid sequences of DR5 (HLYBX88), human tumor necrosis factor receptor 1 (h TNFR1) (SEQ ID NO:3), human Fas protein (SEQ ID NO:4), and the death domain containing receptor 3 (SEQ ID NO:5). The comparison was created with the Megalign program which is contained in the DNA Star suite of programs, using the Clustal method.

FIG. 3 shows an analysis of the DR5 amino acid sequence. Alpha, beta, turn and coil regions; hydrophilicity and hydrophobicity; amphipathic regions; flexible regions; antigenic index and surface probability are shown. In the "Antigenic Index - Jameson-Wolf" graph, amino acid residues about 62 to about 110, about 119 to about 164, about 224 to about 271, and about 275 to about 370 in Figure 1 correspond to the shown highly antigenic regions of the DR5 protein. These highly antigenic fragments in Figure 1 correspond to the following fragments, respectively, in SEQ ID NO:2: amino acid residues from about 11 to about 59, from about 68 to about 113, from about 173 to about 220, and from about 224 to about 319.

FIG. 4 shows the nucleotide sequences (HAPBU13R and HSBBU76R) of two cDNA molecules which are related to the nucleotide sequence shown in Figure 1 (SEQ ID NO:1).

FIG. 5A is a bar graph showing that overexpression of DRS induced apoptosis in MCF7 human breast carcinoma cells. FIG. 5B is a bar graph showing that overexpression of DRS induced apoptosis in human epitheloid carcinoma (HeLa) cells. FIG. 5C is a bar graph showing that DRS-induced apoptosis was blocked by caspase inhibitors, CrmA and z-VAD-fmk, but dominant negative FADD was without effect. FIG. 5D is an immunoblot showing that, like DR4, DRS did not interact with FADD and TRADD *in vivo*. FIG. 5E is a bar graph showing that a dominant negative version of a newly identified FLICE-like molecule, FLICE2 (Vincenz, C. et al., *J. Biol. Chem.* 272:6578 (1997)), efficiently blocked DRS-induced apoptosis, while dominant negative FLICE had only partial effect under conditions it blocked. It also shows that TNFR-1 induced apoptosis effectively.

FIG. 6A is an immunoblot showing that DR5-Fc (as well as DR4 and TRID) specifically bound TRAIL, but not the related cytotoxic ligand TNF α . The bottom panel of Fig. 6A shows the input Fc-fusions present in the binding assays. FIG. 6B is a bar graph showing that DR5-Fc blocked the ability of TRAIL to induce apoptosis. The data (mean \pm SD) shown in Fig. 6B are the percentage of apoptotic nuclei among total nuclei counted ($n=4$). FIG. 6C is a bar graph showing that DR5-Fc had no effect on apoptosis TNF α -induced cell death under conditions where TNFR1-Fc completely abolished TNF α killing.

Detailed Description of the Preferred Embodiments

The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding a DR5 polypeptide having the amino acid sequence shown in FIG. 1 (SEQ ID NO:2), or a fragment of the polypeptide. The DR5 polypeptide of the present invention shares sequence homology with other known death domain containing receptors of the TNFR family including human TNFR- I, DR3 and Fas (FIG. 2). The nucleotide sequence shown in FIG. 1 (SEQ ID NO:1) was obtained by sequencing cDNA clones such as HLYBX88, which was deposited on March 7, 1997 at the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, and given Accession Number 97920. The deposited clone is contained in the pSport 1 plasmid (Life Technologies, Gaithersburg, MD).

Nucleic Acid Molecules

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA

sequencer (such as the Model 373 from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

Using the information provided herein, such as the nucleic acid sequence set out in SEQ ID NO:1, a nucleic acid molecule of the present invention encoding a DR5 polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA as starting material. Illustrative of the invention, the nucleic acid molecule of the invention has been identified in cDNA libraries of the following tissues: primary dendritic cells, endothelial tissue, spleen, chronic lymphocytic leukemia, and human thymus stromal cells.

25 The determined nucleotide sequence of the DRS cDNA of SEQ ID NO:1 contains an open reading frame encoding a protein of about 411 amino acid residues whose initiation codon is at position 130-132 of the nucleotide sequence shown in FIG. 1 (SEQ ID NO.1), with a leader sequence of about 51 amino acid residues. Of known members of the TNF receptor family, the DRS polypeptide of the invention shares the greatest degree of homology with human TNFR1, FAS and DR3 polypeptides shown in Fig. 2, including significant sequence homology over multiple cysteine-rich domains. The homology DRS shows to other death domain containing receptors strongly indicates that DRS is also a death domain containing receptor with the ability to induce apoptosis. 30 DRS has also now been shown to bind TRAIL.

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As indicated, the present invention also provides the manure form(s) of the DR5 protein of the present invention. According to the signal hypothesis, proteins secreted by mammalian cells have a signal or secretory leader sequence

which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Most mammalian cells and even insect cells cleave secreted proteins with the same specificity. However, in some cases, cleavage of a secreted protein is not entirely uniform, which results in two or more mature species on the protein. Further, it has long been known that the cleavage specificity of a secreted protein is ultimately determined by the primary structure of the complete protein, that is, it is inherent in the amino acid sequence of the polypeptide.

Therefore, the present invention provides a nucleotide sequence encoding the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in the host identified as ATCC Deposit No. 97920, and as shown in Figure 1 (SEQ ID NO:2). By the mature DR5 protein having the amino acid sequence encoded by the cDNA clones contained in the host identified as ATCC Deposit No. 97920, is meant the mature form(s) of the DR5 protein produced by expression in a mammalian cell (e.g., COS cells, as described below) of the complete open reading frame encoded by the human DNA sequence of the clone contained in the vector in the deposited host. As indicated below, the mature DR5 having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920, may or may not differ from the predicted "mature" DR5 protein shown in SEQ ID NO:2 (amino acids from about 1 to about 360) depending on the accuracy of the predicted cleavage site based on computer analysis.

Methods for predicting whether a protein has a secretory leader as well as the cleavage point for that leader sequence are available. For instance, the method of McGeoch (*Virus Res.* 3:271-286 (1985)) and von Heinje (*Nucleic Acids Res.* 14:4683-4690 (1986)) can be used. The accuracy of predicting the cleavage points of known mammalian secretory proteins for each of these methods is in the range of 75-80%. von Heinje, *supra*. However, the two methods do not always produce the same predicted cleavage point(s) for a given protein.

In the present case, the predicted amino acid sequence of the complete DR5 polypeptide of the present invention was analyzed by a computer program ("PSORT"). See, K. Nakai and M. Kanehisa, *Genomics* 14:897-911 (1992). PSORT is an expert system for predicting the cellular location of a protein based on the amino acid sequence. As part of this computational prediction of localization, the methods of McGeoch and von Heinje are incorporated. The analysis by the PSORT program predicted the cleavage sites between amino acids 51 and 52 in Figure 1 (-1 and 1 in SEQ ID NO:2). Thereafter, the

complete amino acid sequences were further analyzed by visual inspection, applying a simple form of the (-1,-3) rule of von Heinje. von Heinje, *supra*. Thus, the leader sequence for the DR5 protein is predicted to consist of amino acid residues from about 1 to about 51, underlined in Figure 1 (corresponding to about -51 to about 1 in SEQ ID NO:2), while the predicted mature DR5 protein consists of residues from about 52 to about 411 in Figure 1 (corresponding to about 1 to about 360 in SEQ ID NO:2).

As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

Isolated nucleic acid molecules of the present invention include DR5 DNA molecules comprising an open reading frame (ORF) shown in SEQ ID NO:1; DNA molecules comprising the coding sequence for the mature DR5 protein; and DNA molecules which comprise a sequence substantially different from those described above, but which, due to the degeneracy of the genetic code, still encode the DR5 protein. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate variants.

In another aspect, the invention provides isolated nucleic acid molecules encoding the DR5 polypeptide having an amino acid sequence encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. 97920 on March 7, 1997. In a further embodiment, nucleic acid molecules are provided that encode the mature DR5 polypeptide or the full length DR5 polypeptide lacking the N-terminal methionine. The invention further provides an isolated nucleic acid molecule having the nucleotide sequence shown in SEQ ID NO:1 or

the nucleotide sequence of the DR5 cDNA contained in the above-described deposited clone, or a nucleic acid molecule having a sequence complementary to one of the above sequences. Such isolated molecules, particularly DNA molecules, are useful as probes for gene mapping by *in situ* hybridization with chromosomes, and for detecting expression of the DR5 gene in human tissue, for instance, by Northern blot analysis

The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated DNA molecule having the nucleotide sequence of the deposited cDNA or the nucleotide sequence shown in SEQ ID NO:1 is intended DNA fragments at least about 15 nt, and more preferably at least 20 nt, still more preferably at least about 30 nt, and even more preferably, at least about 40 nt in length, which are useful as diagnostic probes and primers as discussed herein. Of course larger DNA fragments 50-1500 nt in length are also useful according to the present invention, as are fragments corresponding to most, if not all, of the nucleotide sequence of the deposited cDNA or as shown in SEQ ID NO:1. By a fragment at least 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from the nucleotide sequence of the deposited DNA or the nucleotide sequence as shown in SEQ ID NO:1.

Preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising the DR5 extracellular domain (amino acid residues from about 52 to about 184 in FIG. 1 (from about 1 to about 133 in SEQ ID NO:2)); a polypeptide comprising the DR5 transmembrane domain (amino acid residues from about 185 to about 208 in FIG. 1 (from about 134 to about 157 in SEQ ID NO:2)); a polypeptide comprising the DR5 intracellular domain (amino acid residues from about 209 to about 411 in FIG. 1 (from about 158 to about 360 in SEQ ID NO:2)); and a polypeptide comprising the DR5 death domain (amino acid residues from about 324 to about 391 in FIG. 1 (from about 273 to about 340 in SEQ ID NO:2)). Since the location of these domains have been predicted by computer graphics, one of ordinary skill would appreciate that the amino acid residues constituting these domains may vary slightly (e.g., by about 1 to 15 residues) depending on the criteria used to define each domain.

Preferred nucleic acid fragments of the invention encode a full-length DR5 polypeptide lacking the nucleotides encoding the amino-terminal methionine (nucleotides 130-132 in SEQ ID NO:1) as it is known that the methionine is cleaved naturally and such sequences maybe useful in genetically

engineering DRS expression vectors. Polypeptides encoded by such polynucleotides are also contemplated by the invention.

Preferred nucleic acid fragments of the present invention further include nucleic acid molecules encoding epitope-bearing portions of the DRS protein. In particular, such nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising amino acid residues from about 62 to about 110 in Figure 1 (about 11 to about 59 in SEQ ID NO:2); a polypeptide comprising amino acid residues from about 119 to about 164 in Figure 1 (about 68 to about 113 in SEQ ID NO:2); a polypeptide comprising amino acid residues from about 224 to about 271 in Figure 1 (about 173 to about 220 in SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 275 to about 370 in Figure 1 (about 224 to about 319 in SEQ ID NO:2). The inventors have determined that the above polypeptide fragments are antigenic regions of the DRS protein. Methods for determining other such epitope-bearing portions of the DRS protein are described in detail below.

In addition, the invention provides nucleic acid molecules having nucleotide sequences related to extensive portions of SEQ ID NO:1 which have been determined from the following related cDNA clones: HAPBU13R (SEQ ID NO:6) and HSBBU76R (SEQ ID NO:7). The nucleotide sequences of HAPBU13R and HSBBU76R are shown in Figure 4.

Further, the invention includes a polynucleotide comprising any portion of at least about 30 nucleotides, preferably at least about 50 nucleotides, of SEQ ID NO:1 from residue 284 to 1,362, preferably from 284 to 681.

In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the cDNA clones contained in ATCC Deposit No. 97920. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the

reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., the deposited cDNA or the nucleotide sequence as shown in SEQ ID NO:1).

Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3' terminal poly(A) tract of the DR5 cDNA shown in Figure 1 (SEQ ID NO:1)), or to a complementary stretch of T (or U) resides, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

As indicated, nucleic acid molecules of the present invention which encode a DR5 polypeptide may include, but are not limited to the coding sequence for the mature polypeptide, by itself; the coding sequence for the mature polypeptide and additional sequences, such as those encoding a leader or secretary sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing - including splicing and polyadenylation signals, for example - ribosome binding and stability of mRNA; additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, for instance, the polypeptide may be fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell* 37:767 - 778(1984). As discussed below, other such fusion proteins include the DR5 receptor fused to Fc at the N- or C- terminus.

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The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs, or derivatives of the DR5 receptor. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions which may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions, and deletions, which do not alter the properties and activities of the DR5 receptor or portions thereof. Also especially preferred in this regard are conservative substitutions.

Further embodiments of the invention include isolated nucleic acid molecules that are at least 90% identical, and more preferably at least 95%, 96%, 97%, 98% or 99% identical, to (a) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:2; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:2, but lacking the amino terminal methionine; (c) a nucleotide sequence encoding the polypeptide having the amino acid sequence at positions about 1 to about 360 in SEQ ID NO:2; (d) a nucleotide sequence encoding the polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920; (e) a nucleotide sequence encoding the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920; (f) a nucleotide sequence that encodes the DR5 extracellular domain having the amino acid sequence at positions about 1 to about 133 in SEQ ID NO:2, or the DR5 extracellular domain encoded by the cDNA contained in ATCC Deposit No. 97920; (g) a nucleotide sequence that encodes the DR5 transmembrane domain having the amino acid sequence at positions about 134 to about 157 of SEQ ID NO:2, or the DR5 transmembrane domain encoded by the cDNA contained in ATCC Deposit No. 97920; (h) a nucleotide sequence that encodes the DR5 intracellular domain having the amino acid sequence at positions about 158 to about 360 of SEQ ID NO:2, or the DR5 intracellular domain encoded by the cDNA contained in ATCC Deposit No. 97920; (i) a nucleotide sequence that encodes the DR5 death domain

domain having the amino acid sequence at positions about 273 to about 340 of SEQ ID NO:2, or the DR5 death domain encoded by the cDNA contained in ATCC Deposit No. 97920; and (j) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), or (i) above.

- 5 By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding a DR5 polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the DR5 polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5' or 3' terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.
- 10 As a practical matter, whether any particular nucleic acid molecule is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in SEQ ID NO:1 or to the nucleotide sequence of the deposited cDNA clone can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics* 2: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.
- 15 20 25 30 35 The present application is directed to nucleic acid molecules at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in SEQ ID NO:1 or to the nucleic acid sequence of the deposited cDNAs, irrespective of whether they encode a polypeptide having DR5 activity. This is

because even where a particular nucleic acid molecule does not encode a polypeptide having DRS activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having DRS activity include, *inter alia*: (1) isolating the DRS gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the DRS gene, as described in Verma *et al.*, *Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York (1988); and (3) Northern Blot analysis for detecting DRS mRNA expression in specific tissues.

Preferred, however, are nucleic acid molecules having sequences at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in SEQ ID NO:1 or to the nucleic acid sequence of the deposited cDNAs which do, in fact, encode a polypeptide having DRS protein activity. By "a polypeptide having DRS activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to an activity of the DRS protein of the invention (either the full-length protein or, preferably, the mature protein), as measured in a particular biological assay. For example, DRS protein activity can be measured using the cell death assays performed essentially as previously described (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO J* 14, 5579-5588 (1995); A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)) and as set forth in Example 5, below. In MCF7 cells, plasmids encoding full-length DRS or a candidate death domain containing receptor are co-transfected with the pLantern reporter construct encoding green fluorescent protein. Nuclei of cells transfected with DRS will exhibit apoptotic morphology as assessed by DAPI staining. Similar to TNFR-1 and Fas/APO-1 (M. Muzio, *et al.*, *Cell* 85, 817-827 (1996); M. P. Boldin, *et al.*, *Cell* 85, 803-815 (1996); M. Tewari, *et al.*, *J Biol Chem* 270, 3255-60 (1995)), DRS-induced apoptosis is preferably blocked by the inhibitors of ICE-like proteases, CrmA and z-VAD-fmk.

Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the nucleic acid sequence of the deposited cDNA or the nucleic acid sequence shown in SEQ ID NO:1 will encode a polypeptide "having DRS protein activity." In fact, since degenerate variants of these nucleotide sequences

all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having DRS protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J.U. *et al.*, "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science* 247:1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions.

15 ***Polynucleotide Assays***

This invention is also related to the use of the DRS polynucleotides to detect complementary polynucleotides such as, for example, as a diagnostic reagent. Detection of a mutated form of DRS associated with a dysfunction will provide a diagnostic tool that can add or define a diagnosis of a disease or susceptibility to a disease which results from under-expression over-expression or altered expression of DRS or a soluble form thereof, such as, for example, tumors or autoimmune disease.

Individuals carrying mutations in the DRS gene may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR prior to analysis. (Saiki *et al.*, *Nature* 324:163-166 (1986)). RNA or cDNA may also be used in the same ways. As an example, PCR primers complementary to the nucleic acid encoding DRS can be used to identify and analyze DRS expression and mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled DRS RNA or alternatively, radiolabeled DRS antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Sequence differences between a reference gene and genes having mutations also may be revealed by direct DNA sequencing. In addition, cloned

DNA segments may be employed as probes to detect specific DNA segments. The sensitivity of such methods can be greatly enhanced by appropriate use of PCR or another amplification method. For example, a sequencing primer is used with double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotide or by automatic sequencing procedures with fluorescent-tags.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels, with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, *Science* 230:1242 (1985)).

Sequence changes at specific locations also may be revealed by nuclease protection assays, such as RNase and SI protection or the chemical cleavage method (e.g., Cotton *et al.*, *Proc. Natl. Acad. Sci. USA* 85: 4397-4401 (1985)).

Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., restriction fragment length polymorphisms ("RFLP") and Southern blotting of genomic DNA).

In addition to more conventional gel-electrophoresis and DNA sequencing, mutations also can be detected by *in situ* analysis.

Vectors and Host Cells

The present invention also relates to vectors which include DNA molecules of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells can be genetically engineered to incorporate nucleic acid molecules and express polypeptides of the present invention. The polynucleotides may be introduced alone or with other polynucleotides. Such other polynucleotides may be introduced independently, co-introduced or introduced joined to the polynucleotides of the invention.

In accordance with this aspect of the invention the vector may be, for example, a plasmid vector, a single or double-stranded phage vector, a single or

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double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. Viral vectors may be replication competent or replication defective. In the latter case viral propagation generally will occur only in complementing host cells.

5 Preferred among vectors, in certain respects, are those for expression of polynucleotides and polypeptides of the present invention. Generally, such vectors comprise cis-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate trans-
10 acting factors either are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

A great variety of expression vectors can be used to express a polypeptide of the invention. Such vectors include chromosomal, episomal and virus-derived vectors e.g., vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids, all may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides to express a polypeptide in a host may be used for expression in this regard.

15 The DNA sequence in the expression vector is operatively linked to appropriate expression control sequence(s), including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include the phage lambda PL promoter, the *E. coli lac*, *tsp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name just a few of the well-known promoters. In general, expression constructs will contain sites
20 for transcription, initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

25 In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

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Vectors for propagation and expression generally will include selectable markers. Such markers also may be suitable for amplification or the vectors may contain additional markers for this purpose. In this regard, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. Such markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing *E. coli* and other bacteria.

The vector containing the appropriate DNA sequence as described elsewhere herein, as well as an appropriate promoter, and other appropriate control sequences, may be introduced into an appropriate host using a variety of well known techniques suitable to expression therein of a desired polypeptide. Representative examples of appropriate hosts include bacterial cells, such as *E. coli*, Streptomyces and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila S2* and *Spodoptera Sf9* cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and pTrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. These vectors are listed solely by way of illustration of the many commercially available and well known vectors available to those of skill in the art.

Selection of appropriate vectors and promoters for expression in a host cell is a well known procedure and the requisite techniques for expression vector construction, introduction of the vector into the host and expression in the host are routine skills in the art.

The present invention also relates to host cells containing the above-described constructs discussed above. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell; or the host cell can be a prokaryotic cell, such as a bacterial cell.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods.

Such methods are described in many standard laboratory manuals, such as Davis *et al.*, *Basic Methods in Molecular Biology* (1986).

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, region also may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilize proteins. For example, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when the Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as an antigen for immunizations. In drug discovery, for example, human proteins, such as the hIL-5-receptor, have been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. See, D. Bennett *et al.*, *Journal of Molecular Recognition*, 8:52-58 (1995) and K. Johanson *et al.*, *The Journal of Biological Chemistry*, 270:9459-9471 (1995).

The DR5 polypeptides can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and/or purification.

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Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

DRS polynucleotides and polypeptides may be used in accordance with the present invention for a variety of applications, particularly those that make use of the chemical and biological properties of DRS. Among these are applications in treatment of tumors, resistance to parasites, bacteria and viruses, to induce proliferation of T-cells, endothelial cells and certain hematopoietic cells, to treat restenosis, graft vs. host disease, to regulate anti-viral responses and to prevent certain autoimmune diseases after stimulation of DRS by an agonist. Additional applications relate to diagnosis and to treatment of disorders of cells, tissues and organisms. These aspects of the invention are discussed further below.

DRS Polypeptides and Fragments

The invention further provides an isolated DRS polypeptide having the amino acid sequence encoded by the deposited cDNA, or the amino acid sequence in SEQ ID NO:2, or a polypeptide or peptide comprising a portion of the above polypeptides.

It will be recognized in the art that some amino acid sequence of DRS can be varied without significant effect on the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity. Such areas will usually comprise residues which make up the ligand binding site or the death domain, or which form tertiary structures which affect these domains.

Thus, the invention further includes variations of the DRS protein which show substantial DRS protein activity or which include regions of DRS, such as the protein portions discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated above, guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U. et al., *Science* 247:1306-1310 (1990).

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Thus, the fragment, derivative, or analog of the polypeptide of SEQ ID NO:2, or that encoded by the deposited cDNA, may be (i) one in which at least one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue(s)), and more preferably at least one but less than ten conserved amino acid residues) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Of particular interest are substitutions of charged amino acids with another charged amino acids and with neutral or negatively charged amino acids. The latter results in proteins with reduced positive charge to improve the characteristics of the DRS protein. The prevention of aggregation is highly desirable. Aggregation of proteins not only results in a loss of activity but can also be problematic when preparing pharmaceutical formulations, because they can be immunogenic. (Pinckard *et al.*, *Clin Exp. Immunol.* 2:331-340 (1967); Robbins *et al.*, *Diabetes* 36:838-845 (1987); Cleland *et al.* *Crit. Rev. Therapeutic Drug Carrier Systems* 10:307-377 (1993)).

The replacement of amino acids can also change the selectivity of binding to cell surface receptors. Ostade *et al.*, *Nature* 361:266-268 (1993) describes certain mutations resulting in selective binding of TNF-alpha to only one of the two known types of TNF receptors. Thus, the DRS receptor of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation.

As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 1).

TABLE 1. Conservative Amino Acid Substitutions

Aromatic	Phenylalanine Tryptophan Tyrosine
Hydrophobic	Leucine Isoleucine Valine
Polar	Glutamine Asparagine
Basic	Arginine Lysine Histidine
Acidic	Aspartic Acid Glutamic Acid
Small	Alanine Serine Threonine Methionine Glycine

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5 Amino acids in the DRS protein of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, *Science* 244:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as receptor binding or *in vitro*, or *in vitro* proliferative activity. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al.*, *J. Mol. Biol.* 224:899-904 (1992) and de Vos *et al.* *Science* 255:306-312 (1992)).

10 The polypeptides of the present invention are preferably provided in an isolated form. By "isolated polypeptide" is intended a polypeptide removed from its native environment. Thus, a polypeptide produced and/or contained within a recombinant host cell is considered isolated for purposes of the present invention. Also intended as an "isolated polypeptide" are polypeptides that have been purified, partially or substantially, from a recombinant host cell. For example, a recombinantly produced version of the DRS polypeptide can be substantially purified by the one-step method described in Smith and Johnson, *Gene* 67:31-40 (1988).

The polypeptides of the present invention also include the polypeptide encoded by the deposited cDNA including the leader; the mature polypeptide encoded by the deposited cDNA minus the leader (i.e., the mature protein); a polypeptide comprising amino acids about - 51 to about 360 in SEQ ID NO:2; a polypeptide comprising amino acids about - 50 to about 360 in SEQ ID NO:2; a polypeptide comprising amino acids about 1 to about 360 in SEQ ID NO:2; a polypeptide comprising the extracellular domain; a polypeptide comprising the transmembrane domain; a polypeptide comprising the intracellular domain; a polypeptide comprising the extracellular and intracellular domains with all or part of the transmembrane domain deleted; and a polypeptide comprising the death domain; as well as polypeptides which are at least 80% identical, more preferably at least 90% or 95% identical, still more preferably at least 96%, 97%, 98%, or 99% identical to the polypeptides described above, and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a DRS polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the DRS polypeptide. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in Figure 1 (SEQ ID NO:2) or to the amino acid sequence encoded by deposited cDNA clones can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to determine whether a particular

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sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

The polypeptide of the present invention could be used as a molecular weight marker on SDS-PAGE gels or on molecular sieve gel filtration columns using methods well known to those of skill in the art.

The present inventors have discovered that the DRS polypeptide is a 411 residue protein exhibiting three main structural domains. First, the ligand binding domain was identified within residues from about 52 to about 184 in FIG. 1 (amino acid residues from about 1 to about 133 in SEQ ID NO:2). Second, the transmembrane domain was identified within residues from about 185 to about 208 in FIG. 1 (amino acid residues from about 134 to about 157 in SEQ ID NO:2). Third, the intracellular domain was identified within residues from about 209 to about 411 in FIG. 1 (amino acid residues from about 158 to about 360 in SEQ ID NO:2). Importantly, the intracellular domain includes a death domain at residues from about 324 to about 391 (amino acid residues from about 273 to about 340 in SEQ ID NO:2). Further preferred fragments of the polypeptide shown in FIG. 1 include the mature protein from residues about 52 to about 411 (amino acid residues from about 1 to about 360 in SEQ ID NO:2), and soluble polypeptides comprising all or part of the extracellular and intracellular domains but lacking the transmembrane domain.

The invention further provides DRS polypeptides encoded by the deposited cDNA clone including the leader and DRS polypeptide fragments selected from the mature protein, the extracellular domain, the transmembrane domain, the intracellular domain, and the death domain.

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide described herein. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen *et al.*, *Proc. Natl. Acad. Sci. USA* 81:3998-4002 (1983).

As to the selection of peptides or polypeptides bearing an antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G., Shinnick, T. M., Green, N. and Learner, R.A., "Antibodies That React With Predetermined Sites on Proteins," *Science* 219:660-666 (1983). Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. See, for instance, Wilson *et al.*, *Cell* 37:767-778 (1984) at 777. Antigenic epitope-bearing peptides and polypeptides of the invention preferably contain a sequence of at least seven, more preferably at least nine, and most preferably between at least about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate DRS-specific antibodies include: a polypeptide comprising amino acid residues from about 62 to about 110 in Figure 1 (about 11 to about 59 in SEQ ID NO:2); a polypeptide comprising amino acid residues from about 119 to about 164 in Figure 1 (about 68 to about 113 in SEQ ID NO:2); a polypeptide comprising amino acid residues from about 224 to about 271 in Figure 1 (about 173 to about 220 in SEQ ID NO:2); and a polypeptide comprising amino acid residues from about 275 to about 370 in Figure 1 (about 224 to about 319 in SEQ ID NO:2). As indicated above, the inventors have determined that the above polypeptide fragments are antigenic regions of the DRS protein.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means. Houghten, R.A., "General Method for the Rapid Solid-Phase Synthesis of Large Numbers of Peptides: Specificity of Antigen-Antibody Interaction at the Level of Individual Amino Acids," *Proc. Natl. Acad. Sci. USA* 82:5131-5135 (1985). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten *et al.* (1986).

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As one of skill in the art will appreciate, DR5 polypeptides of the present invention and the epitope-bearing fragments thereof described above can be combined with parts of the constant domain of immunoglobulins (IgG), resulting in chimeric polypeptides. These fusion proteins facilitate purification and show an increased half-life *in vivo*. This has been shown, e.g., for chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins (EPA 394,827; Traunecker *et al.*, *Nature* 331:84- 86 (1988)). Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing other molecules than the monomeric DR5 protein or protein fragment alone (Fountoulakis *et al.*, *J Biochem* 270:3958-3964 (1995)).

Polypeptide Assays

The present invention also relates to diagnostic assays such as quantitative and diagnostic assays for detecting levels of DR5 protein, or the soluble form thereof, in cells and tissues, including determination of normal and abnormal levels. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of DR5, or soluble form thereof, compared to normal control tissue samples may be used to detect the presence of tumors, for example. Assay techniques that can be used to determine levels of a protein, such as a DR5 protein of the present invention, or a soluble form thereof, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis, and ELISA assays.

Assaying DR5 protein levels in a biological sample can occur using any art-known method. By "biological sample" is intended any biological sample obtained from an individual, cell line, tissue culture, or other source containing DR5 receptor protein or mRNA. Preferred for assaying DR5 protein levels in a biological sample are antibody-based techniques. For example, DR5 protein expression in tissues can be studied with classical immunohistological methods. (Jalkanen, M. *et al.*, *J. Cell. Biol.* 101:976-985 (1985); Jalkanen, M. *et al.*, *J. Cell. Biol.* 105:3087-3096 (1987)). Other antibody-based methods useful for detecting DR5 protein gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA).

Suitable labels are known in the art and include enzyme labels, such as glucose oxidase, radioisotopes, such as iodine (^{131}I , ^{125}I), carbon (^{14}C), sulphur

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(³⁵S), tritium (³H), indium (¹¹¹In), and technetium (^{99m}Tc), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Therapeutics

5 The Tumor Necrosis Factor (TNF) family ligands are known to be among the most pleiotropic cytokines, inducing a large number of cellular responses, including cytotoxicity, anti-viral activity, immunoregulatory activities, and the transcriptional regulation of several genes (Goeddel, D.V. *et al.*, "Tumor Necrosis Factors: Gene Structure and Biological Activities," *Symp. Quant. Biol.* 51:597-609 (1986), Cold Spring Harbor; Beutler, B., and Cerami, A., *Annu. Rev. Biochem.* 57:505-518 (1988); Old, L.J., *Sci. Am.* 258:59-75 (1988); Fiers, W., *FEBS Lett.* 285:199-224 (1991)). The TNF-family ligands induce such various cellular responses by binding to TNF-family receptors, including the DR5 of the present invention. Cells which express the DR5 polypeptide and are believed to have a potent cellular response to DR5 ligands include primary dendritic cells, endothelial tissue, spleen, chronic lymphocytic leukemia, and human thymus stromal cells. By "a cellular response to a TNF-family ligand" is intended any genotypic, phenotypic, and/or morphologic change to a cell, cell line, tissue, tissue culture or patient that is induced by a TNF-family ligand. As indicated, such cellular responses include not only normal physiological responses to TNF-family ligands, but also diseases associated with increased apoptosis or the inhibition of apoptosis. Apoptosis (programmed cell death) is a physiological mechanism involved in the deletion of peripheral T lymphocytes of the immune system, and its dysregulation can lead to a number of different pathogenic processes (Ameisen, J.C., *AIDS* 8:1197-1213 (1994); Krammer, P.H. *et al.*, *Curr. Opin. Immunol.* 6:279-289 (1994)).

10 Diseases associated with increased cell survival, or the inhibition of apoptosis, include cancers (such as follicular lymphomas, carcinomas with p53 mutations, and hormone-dependent tumors, such as breast cancer, prostate cancer, Kaposi's sarcoma and ovarian cancer); autoimmune disorders (such as systemic lupus erythematosus and immune-related glomerulonephritis, rheumatoid arthritis) and viral infections (such as herpes viruses, pox viruses and adenoviruses), inflammation; graft vs. host disease, acute graft rejection, and chronic graft rejection. Diseases associated with increased apoptosis include AIDS; neurodegenerative disorders (such as Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis, Retinitis pigmentosa, Cerebellar degeneration); myelodysplastic syndromes (such as aplastic anemia),

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ischemic injury (such as that caused by myocardial infarction, stroke and reperfusion injury), toxin-induced liver disease (such as that caused by alcohol), septic shock, cachexia and anorexia.

Thus, in one aspect, the present invention is directed to a method for enhancing apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR5 polypeptide an effective amount of DR5 ligand, analog or an agonist capable of increasing DR5 mediated signaling. Preferably, DR5 mediated signaling is increased to treat a disease wherein decreased apoptosis or decreased cytokine and adhesion molecule expression is exhibited. An agonist can include soluble forms of DR5 and monoclonal antibodies directed against the DR5 polypeptide.

In a further aspect, the present invention is directed to a method for inhibiting apoptosis induced by a TNF-family ligand, which involves administering to a cell which expresses the DR5 polypeptide an effective amount of an antagonist capable of decreasing DR5 mediated signaling. Preferably, DR5 mediated signaling is decreased to treat a disease wherein increased apoptosis or NF κ B expression is exhibited. An antagonist can include soluble forms of DR5 and monoclonal antibodies directed against the DR5 polypeptide.

By "agonist" is intended naturally occurring and synthetic compounds capable of enhancing or potentiating apoptosis. By "antagonist" is intended naturally occurring and synthetic compounds capable of inhibiting apoptosis. Whether any candidate "agonist" or "antagonist" of the present invention can enhance or inhibit apoptosis can be determined using art-known TNF-family ligand/receptor cellular response assays, including those described in more detail below.

One such screening procedure involves the use of melanophores which are transfected to express the receptor of the present invention. Such a screening technique is described in PCT WO 92/01810, published February 6, 1992. Such an assay may be employed, for example, for screening for a compound which inhibits (or enhances) activation of the receptor polypeptide of the present invention by contacting the melanophore cells which encode the receptor with both a TNF-family ligand and the candidate antagonist (or agonist). Inhibition or enhancement of the signal generated by the ligand indicates that the compound is an antagonist or agonist of the ligand/receptor signaling pathway.

Other screening techniques include the use of cells which express the receptor (for example, transfected CHO cells) in a system which measures

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extracellular pH changes caused by receptor activation. For example, compounds may be contacted with a cell which expresses the receptor polypeptide of the present invention and a second messenger response, e.g., signal transduction or pH changes, may be measured to determine whether the potential compound activates or inhibits the receptor.

Another such screening technique involves introducing RNA encoding the receptor into *Xenopus* oocytes to transiently express the receptor. The receptor oocytes may then be contacted with the receptor ligand and a compound to be screened, followed by detection of inhibition or activation of a calcium signal in the case of screening for compounds which are thought to inhibit activation of the receptor.

Another screening technique involves expressing in cells a construct wherein the receptor is linked to a phospholipase C or D. Such cells include endothelial cells, smooth muscle cells, embryonic kidney cells, etc. The screening may be accomplished as hereinabove described by detecting activation of the receptor or inhibition of activation of the receptor from the phospholipase signal.

Another method involves screening for compounds (antagonists) which inhibit activation of the receptor polypeptide of the present invention by determining inhibition of binding of labeled ligand to cells which have the receptor on the surface thereof. Such a method involves transfected a eukaryotic cell with DNA encoding the receptor such that the cell expresses the receptor on its surface and contacting the cell with a compound in the presence of a labeled form of a known ligand. The ligand can be labeled, e.g., by radioactivity. The amount of labeled ligand bound to the receptors is measured, e.g., by measuring radioactivity of the receptors. If the compound binds to the receptor as determined by a reduction of labeled ligand which binds to the receptors, the binding of labeled ligand to the receptor is inhibited.

Further screening assays for agonist and antagonist of the present invention are described in Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267:4304-4307(1992).

Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to a TNF-family ligand. The method involves contacting cells which express the DR5 polypeptide with a candidate compound and a TNF-family ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound.

whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By "assaying a cellular response" is intended qualitatively or quantitatively measuring a cellular response to a candidate compound and/or a TNF-family ligand (e.g., determining or estimating an increase or decrease in T cell proliferation or tritiated thymidine labeling). By the invention, a cell expressing the DRS polypeptide can be contacted with either an endogenous or exogenously administered TNF-family ligand.

Agonist according to the present invention include naturally occurring and synthetic compounds such as, for example, TNF family ligand peptide fragments, transforming growth factor, neurotransmitters (such as glutamate, dopamine, N-methyl-D-aspartate), tumor suppressors (p53), cytolytic T cells and antimetabolites. Preferred agonists include chemotherapeutic drugs such as, for example, cisplatin, doxorubicin, bleomycin, cytosine arabinoside, nitrogen mustard, methotrexate and vincristine. Others include ethanol and amyloid peptide. (*Science* 267:1457-1458 (1995)). Further preferred agonist include polyclonal and monoclonal antibodies raised against the DRS polypeptide, or a fragment thereof. Such agonist antibodies raised against a TNF-family receptor are disclosed in Tartaglia, L.A., et al., *Proc. Natl. Acad. Sci. USA* 88:9292-9296 (1991); and Tartaglia, L.A., and Goeddel, D.V., *J. Biol. Chem.* 267 (7):4304-4307 (1992) See, also, PCT Application WO 94/09137.

Antagonist according to the present invention include naturally occurring and synthetic compounds such as, for example, the CD40 ligand, neutral amino acids, zinc, estrogen, androgens, viral genes (such as Adenovirus *E1B*, Baculovirus *p35* and *IAP*, Cowpox virus *crmA*, Epstein-Barr virus *BHRF1*, *LMP-1*, African swine fever virus *LMW5-HL*, and Herpesvirus *yl 34.5*), calpain inhibitors, cysteine protease inhibitors, and tumor promoters (such as PMA, Phenobarbital, and alpha-Hexachlorocyclohexane).

Other potential antagonists include antisense molecules. Antisense technology can be used to control gene expression through antisense DNA or RNA or through triple-helix formation. Antisense techniques are discussed, for example, in Okano, J. *Neurochem.* 56:560 (1991); *Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression*, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance Lee et al., *Nucleic Acids Research* 6:3073 (1979); Cooney et al., *Science* 241:456 (1988); and Dervan et

al., *Science* 251:1360 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA.

For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of the receptor. The antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into receptor polypeptide. The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed *in vivo* to inhibit production of the receptor.

Further antagonist according to the present invention include soluble forms of DR5, i.e., DR5 fragments that include the ligand binding domain from the extracellular region of the full length receptor. Such soluble forms of the receptor, which may be naturally occurring or synthetic, antagonize DR5 mediated signaling by competing with the cell surface DR5 for binding to TNF-family ligands. Thus, soluble forms of the receptor that include the ligand binding domain are novel cytokines capable of inhibiting apoptosis induced by TNF-family ligands. These are preferably expressed as dimers or trimers, since these have been shown to be superior to monomeric forms of soluble receptor as antagonists, e.g., IgGFc-TNF receptor family fusions. Other such cytokines are known in the art and include Fas B (a soluble form of the mouse Fas receptor) that acts physiologically to limit apoptosis induced by Fas ligand (Hughes, D.P. and Crispe, I.N., *J. Exp. Med.* 182:1395-1401 (1995)).

The term "antibody" (Ab) or "monoclonal antibody" (mAb) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and F(ab')₂ fragments) which are capable of binding an antigen. Fab and F(ab')₂ fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (Wahl *et al.*, *J. Nucl. Med.* 24:316-325 (1983)).

Antibodies according to the present invention may be prepared by any of a variety of methods using DR5 immunogens of the present invention. As indicated, such DR5 immunogens include the full length DR5 polypeptide (which may or may not include the leader sequence) and DR5 polypeptide fragments such as the ligand binding domain, the transmembrane domain, the intracellular domain and the death domain.

Proteins and other compounds which bind the DR5 domains are also candidate agonist and antagonist according to the present invention. Such binding compounds can be "captured" using the yeast two-hybrid system (Fields and Song, *Nature* 340:245-246 (1989)). A modified version of the yeast two-hybrid system has been described by Roger Brent and his colleagues (Gyuris, J. et al., *Cell* 75:791-803 (1993); Zervos, A.S. et al., *Cell* 72:223-232 (1993)). Preferably, the yeast two-hybrid system is used according to the present invention to capture compounds which bind to either the DR5 ligand binding domain or to the DR5 intracellular domain. Such compounds are good candidate agonist and antagonist of the present invention.

By a "TNF-family ligand" is intended naturally occurring, recombinant, and synthetic ligands that are capable of binding to a member of the TNF receptor family and inducing the ligand/receptor signaling pathway. Members of the TNF ligand family include, but are not limited to, DR5 ligands, TRAIL, TNF- α , lymphotoxin- α (LT- α , also known as TNF- β), LT- β (found in complex heterotrimer LT- α 2- β), FasL, CD40, CD27, CD30, 4-IBB, OX40 and nerve growth factor (NGF). Experiments concerning the ability of DR5 to bind TRAIL are described below in Example 6.

Representative therapeutic applications of the present invention are discussed in more detail below. The state of immunodeficiency that defines AIDS is secondary to a decrease in the number and function of CD4 $^{+}$ T-lymphocytes. Recent reports estimate the daily loss of CD4 $^{+}$ T cells to be between 3.5×10^7 and 2×10^9 cells (Wei X. et al., *Nature* 373:117-122 (1995)). One cause of CD4 $^{+}$ T cell depletion in the setting of HIV infection is believed to be HIV-induced apoptosis. Indeed, HIV-induced apoptotic cell death has been demonstrated not only *in vitro* but also, more importantly, in infected individuals (Ameisen, J.C., *AIDS* 8:1197-1213 (1994); Finkel, T.H., and Banda, N.K., *Curr. Opin. Immunol.* 6:605-615(1995); Muro-Cacho, C.A. et al., *J. Immunol.* 154:5555-5566 (1995)). Furthermore, apoptosis and CD4 $^{+}$ T-lymphocyte depletion is tightly correlated in different animal models of AIDS (Brunner, T., et al., *Nature* 373:441-444 (1995); Gougeon, M.L., et al., *AIDS Res. Hum. Retroviruses* 9:553-563 (1993)) and, apoptosis is not observed in those animal models in which viral replication does not result in AIDS (Gougeon, M.L. et al., *AIDS Res. Hum. Retroviruses* 9:553-563 (1993)). Further data indicates that uninfected but primed or activated T lymphocytes from HIV-infected individuals undergo apoptosis after encountering the TNF-family ligand FasL. Using monocytic cell lines that result in death following HIV infection, it has been demonstrated that infection of

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U937 cells with HIV results in the *de novo* expression of FasL and that FasL mediates HIV-induced apoptosis (Badley, A.D. et al., *J. Virol.* 70:199-206 (1996)). Further the TNF-family ligand was detectable in uninfected macrophages and its expression was upregulated following HIV infection resulting in selective killing of uninfected CD4 T-lymphocytes (Badley, A.D. et al., *J. Virol.* 70:199-206 (1996)). Thus, by the invention, a method for treating HIV⁺ individuals is provided which involves administering an antagonist of the present invention to reduce selective killing of CD4 T-lymphocytes. Modes of administration and dosages are discussed in detail below.

In rejection of an allograft, the immune system of the recipient animal has not previously been primed to respond because the immune system for the most part is only primed by environmental antigens. Tissues from other members of the same species have not been presented in the same way that, for example, viruses and bacteria have been presented. In the case of allograft rejection, immunosuppressive regimens are designed to prevent the immune system from reaching the effector stage. However, the immune profile of xenograft rejection may resemble disease recurrence more than allograft rejection. In the case of disease recurrence, the immune system has already been activated, as evidenced by destruction of the native islet cells. Therefore, in disease recurrence the immune system is already at the effector stage. Agonists of the present invention are able to suppress the immune response to both allografts and xenografts because lymphocytes activated and differentiated into effector cells will express the DR5 polypeptide, and thereby are susceptible to compounds which enhance apoptosis. Thus, the present invention further provides a method for creating immune privileged tissues.

DR5 antagonists may be useful for treating inflammatory diseases, such as rheumatoid arthritis, osteoarthritis, psoriasis, septicemia, and inflammatory bowel disease.

In addition, due to lymphoblast expression of DR5, soluble DR5 agonist or antagonist mABs may be used to treat this form of cancer. Further, soluble DR5 or neutralizing mABs may be used to treat various chronic and acute forms of inflammation such as rheumatoid arthritis, osteoarthritis, psoriasis, septicemia, and inflammatory bowel disease.

35 *Modes of Administration*

The agonist or antagonists described herein can be administered *in vitro*, *ex vivo*, or *in vivo* to cells which express the receptor of the present invention. By

5 administration of an "effective amount" of an agonist or antagonist is intended
an amount of the compound that is sufficient to enhance or inhibit a cellular
response to a TNF-family ligand and include polypeptides. In particular, by
administration of an "effective amount" of an agonist or antagonists is intended
10 an amount effective to enhance or inhibit DR5 mediated apoptosis. Of course,
where it is desired for apoptosis is to be enhanced, an agonist according to the
present invention can be co-administered with a TNF-family ligand. One of
ordinary skill will appreciate that effective amounts of an agonist or antagonist
can be determined empirically and may be employed in pure form or in
pharmaceutically acceptable salt, ester or prodrug form. The agonist or
15 antagonist may be administered in compositions in combination with one or
more pharmaceutically acceptable excipients.

15 It will be understood that, when administered to a human patient, the
total daily usage of the compounds and compositions of the present invention
will be decided by the attending physician within the scope of sound medical
judgement. The specific therapeutically effective dose level for any particular
patient will depend upon factors well known in the medical arts.

20 As a general proposition, the total pharmaceutically effective amount of
DR5 polypeptide administered parenterally per dose will be in the range of
about 1 µg/kg/day to 10 mg/kg/day of patient body weight, although, as noted
above, this will be subject to therapeutic discretion. More preferably, this dose
is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01
25 and 1 mg/kg/day for the hormone. If given continuously, the DR5 agonists or
antagonists is typically administered at a dose rate of about 1 µg/kg/hour to
about 50 µg/kg/hour, either by 1-4 injections per day or by continuous
subcutaneous infusions, for example, using a mini-pump. An intravenous bag
solution may also be employed.

30 Dosaging may also be arranged in a patient specific manner to provide a
predetermined concentration of an agonist or antagonist in the blood, as
determined by the RIA technique. Thus patient dosaging may be adjusted to
achieve regular on-going trough blood levels, as measured by RIA, on the order
of from 50 to 1000 ng/ml, preferably 150 to 500 ng/ml.

35 Pharmaceutical compositions are provided comprising an agonist or
antagonist and a pharmaceutically acceptable carrier or excipient, which may be
administered orally, rectally, parenterally, intracistemally, intravaginally,
intraperitoneally, topically (as by powders, ointments, drops or transdermal
patch), buccally, or as an oral or nasal spray. Importantly, by co-administering
an agonist and a TNF-family ligand, clinical side effects can be reduced by

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using lower doses of both the ligand and the agonist. It will be understood that the agonist can be "co-administered" either before, after, or simultaneously with the TNF-family ligand, depending on the exigencies of a particular therapeutic application. By "pharmaceutically acceptable carrier" is meant a non-toxic solid, semisolid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. The term "parenteral" as used herein refers to modes of administration which include intravenous, intramuscular, intraperitoneal, intrasternal, subcutaneous and intraarticular injection and infusion.

Pharmaceutical compositions of the present invention for parenteral injection can comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use.

In addition to soluble DR5 polypeptides, DR5 polypeptide containing the transmembrane region can also be used when appropriately solubilized by including detergents, such as CHAPS or NP-40, with buffer.

Chromosome assays

The nucleic acid molecules of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. The mapping of DNAs to chromosomes according to the present invention is an important first step in correlating those sequences with genes associated with disease.

In certain preferred embodiments in this regard, the cDNA herein disclosed is used to clone genomic DNA of a DR5 gene. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA is then used for *in situ* chromosome mapping using well known techniques for this purpose.

In addition, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes.

Fluorescence *in situ* hybridization ("FISH") of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or

60 bp. For a review of this technique, see Verma *et al.*, *Human Chromosomes: a Manual of Basic Techniques*, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, *Mendelian Inheritance in Man*, available on line through Johns Hopkins University, Welch Medical Library. The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

Example 1

Expression and Purification in E. coli

The DNA sequence encoding the mature DRS protein in the deposited cDNA clone (ATCC No. 97920) is amplified using PCR oligonucleotide primers specific to the amino terminal sequences of the DRS protein and to vector sequences 3' to the gene. Additional nucleotides containing restriction sites to facilitate cloning are added to the 5' and 3' sequences respectively.

The following primers are used for expression of DRS extracellular domain in *E. coli*. The 5' primer has the sequence 5'-CGCCCATGGAGTCTGCTCTGATCAC-3' (SEQ ID NO:8) and contains the underlined NcoI site; and the 3' primer has the sequence 5'-CGCAAGCTTTAGCCTGATTCTTGTGGAC-3' (SEQ ID NO:9) and contains the underlined HindIII site.

The restriction sites are convenient to restriction enzyme sites in the bacterial expression vector pQE60, which are used for bacterial expression in this example. (Qiagen, Inc. 9259 Eton Avenue, Chatsworth, CA, 91311). pQE60 encodes ampicillin antibiotic resistance ("Amp") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, and a ribosome binding site ("RBS").

The amplified DRS DNA and the vector pQE60 both are digested with NcoI and HindIII and the digested DNAs are then ligated together. Insertion of

the DR5 protein DNA into the restricted pQE60 vector places the DR5 protein coding region downstream of and operably linked to the vector's IPTG-inducible promoter and in-frame with an initiating AUG appropriately positioned for translation of DR5 protein.

- 5 The ligation mixture is transformed into competent *E. coli* cells using standard procedures. Such procedures are described in Sambrook *et al.*, Molecular Cloning: a Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses lac repressor and confers kanamycin resistance ("Kan"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing DR5 protein, is available commercially from Qiagen, *supra*.
- 10 Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR, and DNA sequencing.
- 15 Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 µg/ml) and kanamycin (25 µg/ml). The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:100 to 1:250. The cells are grown to an optical density at 600nm ("OD₆₀₀") of between 0.4 and 0.6. Isopropyl-B-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from *lac* repressor sensitive promoters, by inactivating the *lacI* repressor. Cells subsequently are incubated further for 3 to 4 hours.
- 20 Cells then are harvested by centrifugation and disrupted, by standard methods. Inclusion bodies are purified from the disrupted cells using routine collection techniques, and protein is solubilized from the inclusion bodies into 8M urea. The 8M urea solution containing the solubilized protein is passed over a PD-10 column in 2X phosphate-buffered saline ("PBS"), thereby removing the urea, exchanging the buffer and refolding the protein. The protein is purified by a further step of chromatography to remove endotoxin. Then, it is sterile filtered. The sterile filtered protein preparation is stored in 2X PBS at a concentration of 95 µ/ml.
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*Example 2**Expression in Mammalian Cells*

A typical mammalian expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g. RSV, HTLV, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular signals can also be used (e.g. the human actin promoter). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC67109). Mammalian host cells that could be used include, human Hela 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the gene of interest can be expressed in stable cell lines that contain the gene integrated into a chromosome. Co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells.

The transfected gene can also be amplified to express large amounts of the encoded protein. The dihydrofolate reductase (DHFR) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy *et al.*, *Biochem. J.* 227:277-279 (1991); Bebbington *et al.*, *BioTechnology* 10:169-175 (1992)). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) cells are often used for the production of proteins.

The expression vectors pCl and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen *et al.*, *Molecular and Cellular Biology* 5:438-447 (March 1985)), plus a fragment of the CMV-enhancer (Boshart *et al.*, *Cell* 41:521-530 (1985)). Multiple cloning sites, e.g. with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of

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interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene.

Cloning and Expression in CHO Cells

The vector pC4 is used for the expression of the DR5 polypeptide. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary- or other cells lacking dihydrofolate activity that are transfected with these plasmids, can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate (MTX). The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented (see, e.g., Alt, F. W., Kellems, R. M., Bertino, J. R., and Schimke, R. T., *J. Biol. Chem.* 253:1357-1370 (1978); Hamlin, J. L. and Ma, C., *Biochem. et Biophys. Acta* 1097:107-143 (1990); Page, M. J. and Sydenham, M. A. 1991, *Biotechnology* 9:64-68(1991)). Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach may be used to develop cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently, when the methotrexate is withdrawn, cell lines are obtained which contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains, for expressing the gene of interest, the strong promoter of the long terminal repeat (LTR) of the Rous Sarcoma Virus (Cullen et al., *Molecular and Cellular Biology* 5:438-447(March 1985), plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV) (Boshart et al., *Cell* 41:521-530 (1985)). Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: BamHI, Xba I, and Asp718. Behind these cloning sites the plasmid contains the 3' intron and polyadenylation site of the rat preproinsulin gene. Other high efficiency promoters can also be used for expression, e.g., the human β -actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLV. Clontech's Tet-Off and Tet-On gene expression systems and similar systems

can be used to express the DRS polypeptide in a regulated way in mammalian cells (Gossen, M., & Bujard, H., *Proc. Natl. Acad. Sci. USA* 89:5547-5551 (1992). For the polyadenylation of the mRNA, other signals, e.g., from the human growth hormone or globin genes, can be used as well.

Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418, or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with the restriction enzyme BamHI and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel.

The DNA sequence encoding the complete polypeptide is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the desired portion of the gene. The 5' primer containing the underlined BamHI site, a Kozak sequence, and an AUG start codon, has the following sequence: 5' CGCGGATCCGCCATCATGGAAACAACGGGGACAGAAC 3' (SEQ ID NO:10). The 3' primer, containing the underlined Asp718 site, has the following sequence: 5' CGCGGTTACCTTAGGACATGGCAGAGTC 3' (SEQ ID NO:11).

The amplified fragment is digested with the endonuclease BamHI and Asp718 and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene are used for transfection. Five μ g of the expression plasmid pC4 is cotransfected with 0.5 μ g of the plasmid pSVneo using the lipofectin method (Felgner et al., *supra*). The plasmid pSV2-neo contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 1 mg/ml G418. After about 10-14 days, single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM,

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400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 μ M, 2 μ M, 5 μ M, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200 μ M. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

Cloning and Expression in COS Cells

10 The expression plasmid, pDR5-HA, is made by cloning a cDNA encoding the soluble extracellular domain of the DR5 protein into the expression vector pcDNAI/Amp or pcDNAIII (which can be obtained from Invitrogen, Inc.). The expression vector pcDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cells; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 and a polyadenylation signal arranged so that a cDNA can be conveniently placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker. A DNA fragment encoding the extracellular domain of the DR5 polypeptide and a HA tag fused in frame to its 3' end is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the CMV promoter. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson et al., *Cell* 37: 767 (1984). The fusion of the HA tag to the target protein allows easy detection and recovery of the recombinant protein with an antibody that recognizes the HA epitope.

15 20 25 30 The plasmid construction strategy is as follows. The DR5 cDNA of the deposited clone is amplified using primers that contain convenient restriction sites, much as described above for construction of vectors for expression of DR5 in *E. coli*.

To facilitate detection, purification and characterization of the expressed DR5, one of the primers contains a hemagglutinin tag ("HA tag") as described above.

35 Suitable primers include the following, which are used in this example. The 5' primer, containing the underlined BamHI site has the following sequence: 5' CGCGGATCCGCCCATCATGGAAACACGGGGACAGAAC 3' (SEQ ID NO:10). The 3' primer, containing the underlined Asp718 restriction

sequence has the following sequence: 5'
CGCGGTACCTTAGCCTGATTCTTGAC 3' (SEQ ID NO:12).

The PCR amplified DNA fragment and the vector, pcDNA1/Amp, are digested with BamHI and Asp718 and then ligated. The ligation mixture is transformed into *E. coli* strain SURE (available from Stratagene Cloning Systems, 11099 North Torrey Pines Road, La Jolla, CA 92037), and the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis or other means for the presence of the fragment encoding the extracellular domain of the DRS polypeptide

For expression of recombinant DRS, COS cells are transfected with an expression vector, as described above, using DEAE-DEXTRAN, as described, for instance, in Sambrook et al., *Molecular Cloning: a Laboratory Manual*, Cold Spring Laboratory Press, Cold Spring Harbor, New York (1989). Cells are incubated under conditions for expression of DRS by the vector.

Expression of the DRS-HA fusion protein is detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow et al., *Antibodies: A Laboratory Manual*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988). To this end, two days after transfection, the cells are labeled by incubation in media containing 35 S-cysteine for 8 hours. The cells and the media are collected, and the cells are washed and lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson et al., cited above. Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then are analyzed by SDS-PAGE and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

The primer sets used for expression in this example are compatible with pC4 used for CHO expression in this example, pcDNA1/Amp for COS expression in this example, and pA2 used for baculovirus expression in the following example. Thus, for example, the complete DRS encoding fragment amplified for CHO expression could also be ligated into pcDNA1/Amp for COS expression or pA2 for baculovirus expression.

Example 3

Cloning and expression of the soluble extracellular domain of DRS in a baculovirus expression system

In this illustrative example, the plasmid shuttle vector pA2 is used to insert the cloned DNA encoding the complete protein, including its naturally associated signal sequence, into a baculovirus to express the DRS protein, using standard methods, such as those described in Summers *et al.*, *A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedron promoter of the *Autograph californica nuclear polyhedrosis virus* (ACMNPV) followed by convenient restriction sites. For easy selection of recombinant virus, the plasmid contains the beta-galactosidase gene from *E. coli* under control of a weak Drosophila promoter in the same orientation, followed by the polyadenylation signal of the polyhedrin gene. The inserted genes are flanked on both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that express the cloned polynucleotide. Many other baculovirus vectors could be used in place of pA2, such as pAc373, pVL941 and pAcIM1 provided, as one skilled in the art would readily appreciate, that construction provides appropriately located signals for transcription, translation, secretion, and the like, such as an in-frame AUG and a signal peptide, as required. Such vectors are described, for example, in Luckow *et al.*, *Virology* 170:31-39 (1989).

The cDNA sequence encoding the soluble extracellular domain of DRS protein in the deposited clone (ATCC No. 97920) is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer for DRS has the sequence 5' CGCGGATCCGCCATCATGGAAACAACGGGGACAGAAC 3' (SEQ ID NO:10) containing the underlined BamHI restriction enzyme site. Inserted into an expression vector, as described below, the 5' end of the amplified fragment encoding DRS provides an efficient cleavage signal peptide. An efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., *J. Mol. Biol.* 196:947-950 (1987) is appropriately located in the vector portion of the construct.

The 3' primer for DRS has the sequence 5' CGCGGTACCTTAGCCTGATTCTTGTGGAC 3' (SEQ ID NO:12) containing the underlined Asp718 restriction followed by nucleotides

complementary to the DRS nucleotide sequence in FIG. 1, followed by the stop codon.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.) The fragment then is digested with BamHI and Asp718 and again is purified on a 1% agarose gel. This fragment is designated "F1."

The plasmid is digested with the restriction enzymes Bam HI and Asp718 and optionally can be dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("Geneclean" BIO 101 Inc., La Jolla, Ca.). The vector DNA is designated herein "V1."

Fragment F1 and the dephosphorylated plasmid V1 are ligated together with T4 DNA ligase. *E. coli* HB101 cells, or other suitable *E. coli* hosts such as XL-1 Blue (Stratagene Cloning Systems, La Jolla, CA) cells, are transformed with the ligation mixture and spread on culture plates. Bacteria are identified that contain the plasmid with the human DRS using the PCR method, in which one of the primers that is used to amplify the gene and the second primer is from well within the vector so that only those bacterial colonies containing the DRS gene fragment will show amplification of the DNA. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pBac DRS.

5 µg of the plasmid pBac DRS is co-transfected with 1.0 µg of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA", Pharmingen, San Diego, CA.), using the lipofectin method described by Felgner *et al.*, *Proc. Natl. Acad. Sci. USA* 84:7413-7417 (1987). 1 µg of BaculoGold™ virus DNA and 5 µg of the plasmid pBac DRS are mixed in a sterile well of a microliter plate containing 50 µl of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 µl Lipofectin plus 90 µl Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27°C. After 5 hours, the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27°C for four days.

After four days, the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, cited above. An agarose gel

with "Blue Gal" (Life Technologies Inc., Gaithersburg, MD) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, MD, pages 9-10). After appropriate incubation, blue stained plaques are picked with the tip of a micropipettor (e.g. Eppendorf). The agar containing the recombinant viruses is then resuspended in a microcentrifuge tube containing 200 μ l of Grace's medium and the suspension containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later, the supernatants of these culture dishes are harvested and then they are stored at 4°C. The recombinant virus is called V-DR5.

To verify expression of the DR5 gene, Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus V-DR5 at a multiplicity of infection ("MOI") of about 2 (about 1 to about 3). Six hours later, the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Gaithersburg, MD). If radiolabeled proteins are desired, 42 hours later, 5 μ Ci of 35 S-methionine and 5 μ Ci 35 S cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then they are harvested by centrifugation. The proteins in the supernatant as well as the intracellular proteins are analyzed by SDS-PAGE followed by autoradiography (if radiolabeled). Microsequencing of the amino acid sequence of the amino terminus of purified protein may be used to determine the amino terminal sequence of the mature protein and thus the cleavage point and length of the secretory signal peptide.

Example 4

Tissue distribution of DR5 gene expression

Northern blot analysis was carried out to examine DR5 gene expression in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. A cDNA probe containing the entire nucleotide sequence of the DR5 protein (SEQ ID NO:1) was labeled with 32 P using the rediprimeTM DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe was purified using a CHROMA SPIN-100TM column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe was then used to examine various human tissues for DR5 mRNA.

Multiple Tissue Northern (MTN) blots containing various human tissues (H) or human immune system tissues (IM) were obtained from Clontech (Palo Alto, CA) and examined with labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots were mounted and exposed to film at -70°C overnight. The films were developed according to standard procedures. Expression of DRS was detected in heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, spleen, thymus, prostate, testis, uterus, small intestine, colon, peripheral blood leukocytes (PBLs), lymph node, bone marrow, and fetal liver.

Expression of DRS was also assessed by Northern blot in the following cancer cell lines, HL60 (promyelocytic leukemia), Hela cell S3, K562 (chronic myelogenous leukemia), MOLT4 (lymphoblast leukemia), Raji (Burkitt's lymphoma), SW480 (colorectal adenocarcinoma), A549 (lung carcinoma), and G361 (melanoma), and was detected in all of the cell lines tested.

Example 5

DRS Induced Apoptosis in Mammalian Cells

Overexpression of Fas/APO-1 and TNFR-1 in mammalian cells mimics receptor activation (M. Muzio *et al.*, *Cell* 85, 817-827 (1996); M. P. Boldin *et al.*, *Cell* 85, 803-815 (1996)). Thus, this system was utilized to study the functional role of DRS in inducing apoptosis. This example demonstrates that overexpression of DRS induced apoptosis in both MCF7 human breast carcinoma cells and in human epithelial carcinoma (Hela) cells.

Experimental Design

Cell death assays were performed essentially as previously described (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol Chem* 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO J* 14, 5579-5588 (1995); A.M. Chinnaiyan, *et al.*, *J Biol Chem* 271, 4961-4965 (1996)). Briefly, MCF-7 human breast carcinoma clonal cell lines and Hela cells were co-transfected with vector, DRS, DR Δ (52-411), or TNFR-1, together with a beta-galactosidase reporter construct.

MCF7 and Hela cells were transfected using the lipofectamine procedure (GIBCO-BRL), according to the manufacturer's instructions. 293 cells were transfected using CaPO₄ precipitation. Twenty-four hours following transfection, cells were fixed and stained with X-Gal as previously described (A.M. Chinnaiyan, *et al.*, *Cell* 81, 505-12 (1995); M.P. Boldin, *et al.*, *J Biol*

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Chem 270, 7795-8 (1995); F.C. Kischkel, *et al.*, *EMBO J.* 14, 5579-5588 (1995)), and examined microscopically. The data (mean \pm SD) presented in Figure 5 represents the percentage of round, apoptotic cells as a function of total beta-galactosidase positive cells (n=3). Overexpression of DR5 induced apoptosis in both MCF7 (Fig. 5A) and HeLa cells (Fig. 5B).

MCF7 cells were also transfected with a DR5 expression construct in the presence of z-VAD-fmk (20 μ l)(Enzyme Systems Products, Dublin, CA) or co-transfected with a three-fold excess of CrmA (M. Tewari *et al.*, *J Biol Chem* 270: 3255-60 (1995)), or FADD-DN expression construct, or vector alone. The data presented in Fig. 5C shows that apoptosis induced by DR5 was attenuated by caspase inhibitors ,but not by dominant negative FADD.

As depicted in Fig. 5D, DR5 did not associate with FADD or TRADD *in vivo*. 293 cells were co-transfected with the indicated expression constructs using calcium phosphate precipitation. After transfection (at 40 hours), cell lysates were prepared and immunoprecipitated with Flag M2 antibody affinity gel (IBI, Kodak), and the presence of FADD or myc-tagged TRADD (myc-TRADD) was detected by immunoblotting with polyclonal antibody to FADD or horseradish peroxidase (HRP) conjugated antibody to myc (BMB)(Baker, S.J. *et al.*, *Oncogene* 12:1 (1996); Chinnaiyan, A.M. *et al.*, *Science* 274:990 (1996)).

As depicted in Fig. 5E, FLICE 2-DN blocks DR5-induced apoptosis. 293 cells were co-transfected with DR5 or TNFR-1 expression construct and a fourfold excess of CrmA, FLICE-DN, FLICE 2-DN, or vector alone in the presence of a beta-galactosidase reporter construct as indicated. Cells were stained and examined 25-30 hours later.

Results

Overexpression of DR5, induced apoptosis in both MCF7 human breast carcinoma cells (Fig. 5A) and in human epithelial carcinoma (HeLa) cells (Fig. 5B). Most of the transfected cells displayed morphological changes characteristic of cells undergoing apoptosis (Earnshaw, W.C., *Curr. Biol.* 7:337 (1995)), becoming rounded, condensed and detaching from the dish. Deletion of the death domain abolished killing ability. Like DR4, DR5-induced apoptosis was blocked by caspase inhibitors, CrmA and z-VAD-fmk, but dominant negative FADD was without effect (Fig. 5C). Consistent with this, DR5 did not interact with FADD and TRADD *in vivo* (Fig. 5D). A dominant negative version of a newly identified FLICE-like molecule, FLICE2 (Vincenz, C. *et al.*, *J. Biol. Chem.* 272:6578 (1997)), efficiently blocked DR5-induced

apoptosis, while dominant negative FLICE had only partial effect under conditions it blocked. TNFR-1 induced apoptosis effectively (Fig. 5E). Taken together, the evidence suggests that DR5 engages an apoptotic program that involves activation of FLICE2 and downstream caspases, but is independent of FADD.

Example 6

The Extracellular Domain of DR5 Binds the Cytotoxic Ligand--TRAIL and Blocks TRAIL-Induced Apoptosis

As discussed above, TRAIL/Apo2L is a cytotoxic ligand that belongs to the tumor necrosis factor (TNF) ligand family and induces rapid cell death of many transformed cell lines, but not normal tissues, despite its death domain containing receptor, DR4, being expressed on both cell types. This example shows that the present receptor, DR5, also binds TRAIL.

Given the similarity of the extracellular ligand binding cysteine-rich domains of DR5 and DR4, the present inventors theorized that DR5 would also bind TRAIL. To confirm this, the soluble extracellular ligand binding domains of DR5 were expressed as fusions to the Fc portion of human immunoglobulin (IgG).

As shown in Fig. 6A, DRS-Fc specifically bound TRAIL, but not the related cytotoxic ligand TNF α . In this experiment, the Fc-extracellular domains of DRS, DR4, TRID, or TNFR1 and the corresponding ligands were prepared and binding assays performed as described in Pan *et al.*, *Science* 276:111 (1997). The respective Fc-fusions were precipitated with protein G-Sepharose and co-precipitated soluble ligands were detected by immunoblotting with anti-Flag (Babco) or anti-myc-HRP (BMB). The bottom panel of Fig. 6A shows the input Fc-fusions present in the binding assays.

Additionally, DR5-Fc blocked the ability of TRAIL to induce apoptosis (Fig. 6B). MCF7 cells were treated with soluble TRAIL (200 ng/ml) in the presence of equal amounts of Fc-fusions or Fc alone. Six hours later, cells were fixed and examined as described in Pan et al., *Id*. The data (mean \pm SD) shown in Fig. 6B are the percentage of apoptotic nuclei among total nuclei counted ($n=4$).

Finally, DR5-Fc had no effect on apoptosis TNF α -induced cell death under conditions where TNFR1-Fc completely abolished TNF α killing (Fig 6C). MCF7 cells were treated with TNF α (40 ng/ml; Genentech, Inc.) in the presence of equal amounts of Fc-fusions or Fc alone. Nuclei were stained and examined 11-15 hours later.

The new identification of DR5 as a receptor for TRAIL adds further complexity to the biology of TRAIL-initiated signal transduction.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples.

Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosures of all patents, patent applications, and publications referred to herein are hereby incorporated by reference.

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WHAT IS CLAIMED IS:

1. An isolated nucleic acid molecule nucleic acid molecule comprising a polynucleotide having a nucleotide sequence at least 95% identical to a sequence selected from the group consisting of:
- (a) a nucleotide sequence encoding a polypeptide comprising amino acids from about -51 to about 360 in SEQ ID NO:2;
- (b) a nucleotide sequence encoding a polypeptide comprising amino acids from about -50 to about 360 in SEQ ID NO:2;
- (c) a nucleotide sequence encoding a polypeptide comprising amino acids from about 1 to about 360 in SEQ ID NO:2;
- (d) a nucleotide sequence encoding a polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;
- (e) a nucleotide sequence encoding the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;
- (f) a nucleotide sequence encoding the DR5 extracellular domain;
- (g) a nucleotide sequence encoding the DR5 transmembrane domain;
- (h) a nucleotide sequence encoding the DR5 intracellular domain;
- (i) a nucleotide sequence encoding the DR5 death domain; and
- (j) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), or (i) above.
2. The nucleic acid molecule of claim 1, wherein said polynucleotide has the nucleotide sequence in SEQ ID NO:1.
3. The nucleic acid molecule of claim 1, wherein said polynucleotide has the nucleotide sequence in SEQ ID NO:1 encoding the DR5 polypeptide having the amino acid sequence in SEQ ID NO:2.
4. The nucleic acid molecule of claim 1, wherein said polynucleotide has the nucleotide sequence in SEQ ID NO:1 encoding the mature DR5 polypeptide having the amino acid sequence in SEQ ID NO:2.
5. The nucleic acid molecule of claim 1, wherein said polynucleotide has the complete nucleotide sequence of the cDNA clone contained in ATCC Deposit No. 97920.

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6. The nucleic acid molecule of claim 1, wherein said polynucleotide has the nucleotide sequence encoding the DRS polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920.

5

7. The nucleic acid molecule of claim 1, wherein said polynucleotide has the nucleotide sequence encoding the mature DRS polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920.

10

15

8. An isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having a nucleotide sequence identical to a nucleotide sequence in (a), (b), (c), (d), (e), (f), (g), (h), (i), or (j) of claim 1, wherein said polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues.

20

25

9. An isolated nucleic acid molecule comprising a polynucleotide which encodes the amino acid sequence of an epitope-bearing portion of a DRS polypeptide having an amino acid sequence in (a), (b), (c), (d), (e), (f), (g), (h), or (i) of claim 1.

30

35

10. The isolated nucleic acid molecule of claim 9, which encodes an epitope-bearing portion of a DRS polypeptide selected from the group consisting of: a polypeptide comprising amino acid residues from about 11 to about 59 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 68 to about 113 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 173 to about 220 in SEQ ID NO:2; and a polypeptide comprising amino acid residues from about 224 to about 319 in SEQ ID NO:2.

11. The isolated nucleic acid molecule of claim 1, which encodes the DRS receptor extracellular domain.

12. The isolated nucleic acid molecule of claim 1, which encodes the DRS receptor transmembrane domain.

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13. The isolated nucleic acid molecule of claim 1, which encodes the DR5 receptor intracellular domain

5 14. A method for making a recombinant vector comprising inserting an isolated nucleic acid molecule of claim 1 into a vector.

15. A recombinant vector produced by the method of claim 14.

10 16. A method of making a recombinant host cell comprising introducing the recombinant vector of claim 15 into a host cell.

17. A recombinant host cell produced by the method of claim 16.

15 18. A recombinant method for producing a DR5 polypeptide, comprising culturing the recombinant host cell of claim 17 under conditions such that said polypeptide is expressed and recovering said polypeptide.

19. An isolated DR5 polypeptide comprising an amino acid sequence at least 95% identical to a sequence selected from the group consisting of:

- 20 (a) amino acids from about -51 to about 360 in SEQ ID NO:2;
(b) amino acids from about -50 to about 360 in SEQ ID NO:2;
(c) amino acids from about 1 to about 360 in SEQ ID NO:2;
25 (d) the amino acid sequence of the DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;
(e) the amino acid sequence of the mature DR5 polypeptide having the amino acid encoded by the cDNA clone contained in ATCC Deposit No. 97920;
(f) the amino acid sequence of the DR5 extracellular domain;
(g) the amino acid sequence of the DR5 transmembrane domain;
30 (h) the amino acid sequence of the DR5 intracellular domain;
(i) the amino acid sequence of the DR5 death domain;
(j) the amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a), (b), (c), (d), (e), (f), (g), (h), (i), or (j).

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- 5
20. An isolated polypeptide comprising an epitope-bearing portion of the DRS protein, wherein said portion is selected from the group consisting of: a polypeptide comprising amino acid residues from about 11 to about 59 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 68 to about 113 in SEQ ID NO:2; a polypeptide comprising amino acid residues from about 173 to about 220 in SEQ ID NO:2; and a polypeptide comprising amino acid residues from about 224 to about 319 in SEQ ID NO:2.
- 10
21. An isolated antibody that binds specifically to a DRS polypeptide of claim 19.
- 15
22. An isolated nucleic acid molecule comprising a polynucleotide having a sequence at least 95% identical to a sequence selected from the group consisting of:
- (a) the nucleotide sequence of clone HAPBU13R (SEQ ID NO:6);
 - (b) the nucleotide sequence of clone HSBBU76R (SEQ ID NO:7);
 - (c) the nucleotide sequence of a portion of the sequence shown in Figure 1 (SEQ ID NO:1) wherein said portion comprises at least 50 contiguous nucleotides from nucleotide 284 to 1,362; and
 - 20
 - (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b) or (c) above.
- 25
23. An isolated nucleic acid molecule comprising a polynucleotide encoding a DRS receptor polypeptide wherein, except for at least one conservative amino acid substitution, said polypeptide has a sequence selected from the group consisting of:
- (a) a nucleotide sequence encoding a polypeptide comprising amino acids from about -51 to about 360 in SEQ ID NO:2;
 - 30
 - (b) a nucleotide sequence encoding a polypeptide comprising amino acids from about -50 to about 360 in SEQ ID NO:2;
 - (c) a nucleotide sequence encoding a polypeptide comprising amino acids from about 1 to about 360 in SEQ ID NO:2;
 - 35
 - (d) a nucleotide sequence encoding a polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;
 - (e) a nucleotide sequence encoding the mature DRS polypeptide having the amino acid sequence encoded by the cDNA clone

contained in ATCC Deposit No. 97920;

(f) a nucleotide sequence encoding the DR5 extracellular domain;

5 (g) a nucleotide sequence encoding the DR5 transmembrane domain;

(h) a nucleotide sequence encoding the DR5 intracellular domain;

10 (i) a nucleotide sequence encoding the DR5 receptor extracellular and intracellular domains with all or part of the transmembrane domain deleted;

(j) a nucleotide sequence encoding the DR5 death domain; and

15 (k) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g), (h), (i), or (j).

24. An isolated DR5 receptor polypeptide wherein, except for at least one conservative amino acid substitution, said polypeptide has a sequence selected from the group consisting of:

20 (a) amino acids from about -51 to about 360 in SEQ ID NO:2;

(b) amino acids from about -50 to about 360 in SEQ ID NO:2;

(c) amino acids from about 1 to about 360 in SEQ ID NO:2;

25 (d) the amino acid sequence of the DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;

(e) the amino acid sequence of the mature DR5 polypeptide having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97920;

30 (f) the amino acid sequence of the DR5 receptor extracellular domain;

(g) the amino acid sequence of the DR5 receptor transmembrane domain;

35 (h) the amino acid sequence of the DR5 receptor intracellular domain;

(i) the amino acid sequence of the DR5 receptor extracellular and intracellular domains with all or part of the transmembrane domain deleted;

(j) the amino acid sequence of the DR5 receptor death

domain; and

(k) the amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a), (b), (c), (d), (e), (f), (g), (h), (i), or (j).

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Death Domain Containing Receptor 5

Abstract

5 The present invention relates to novel Death Domain Containing
Receptor-5 (DR5) proteins which are members of the tumor necrosis factor
(TNF) receptor family, and have now been shown to bind TRAIL. In
particular, isolated nucleic acid molecules are provided encoding the human
DR5 proteins. DR5 polypeptides are also provided as are vectors, host cells
10 and recombinant methods for producing the same. The invention further relates
to screening methods for identifying agonists and antagonists of DR5 activity.

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Figure 1

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10		30	50
CACGGCTCCGGCGGGCGGAGAACCCGCAATTTGC	CCCACAA	AAATACACCGA	
70	90	110	
CGATGCCGATCTACTTTAAGGGTGA	AAACCCACGGGCTGAGAGACTATAAGAGCGTTC		
130	150	170	
CCTACCGCCATGAAACAACGGGACAGAACGCC	CCGGCCTMC	GGGGCCGGAAAAGG	
M E O R G O N A P A A S G A R K R			
190	210	230	
CACGGCCCAGGACCAGGGAGGCGCGGGAGCCAGG	CCCTGGGCCCCGGGTCCCAAGACC		
H G P G P R E A R G A R P G P R V P K T			
250	270	290	
CTTGTGCTCGTTGCGCCGGTCCCTGCTGTTGGTCTCAGCTGAGTCTGCTCTGATCACC			
L V L V V A A V L L L V S A E S A L I T			
310	330	350	
CAACAGACCTAGCTCCCCAGCAGAGCGGCCCCACAA	ACAAAGAGGTCCAGCCCCCTCA		
Q Q D L A P Q Q R A A P Q Q K R S S P S			
370	390	410	
GAGGGATTGTGTCACCTGGACACCATATCTCAGAAGACGGTAGAGATTG	CATCTCCTGC		
E G L C P P G H H I S E D G R D C I S C			
430	450	470	
AAATATGGACAGGACTATAGCACTCACTGGAA	TGACCTCCTTCTGCTTGCGCTGCACC		
K Y G Q D Y S T H W N D L L F C L R C T			
490	510	530	
AGGTGTGATTCAAGGTGAAGTGGAGCTAAGTCCCTGCACCA	CCACGACAGAACACAGTGTG		
R C D S G E V E L S P C T T T R N T V C			
550	570	590	
CACTGCGAAGAAGGCACCTTCCGGGAAGAAGATTCTCCTGAGATGTG	CCGGAAAGTGCCGC		
Q C E E G T P R E E D S P E M C R K C R			
610	630	650	
ACAGGGTGTCCCAGAGGGATGGTCAGGTGGTATTGTACACCC	CTGGAGTGACATCGAA		
T G C P R G M V K V G D C T P W S D I E			
670	690	710	
TGTGTCACAAAGAACATCAGGCATCATCAGG	GTACAGTTGCA	CCGGTAGTC	TGATT
C V H K E S G I I G V T V A A V V L I			
730	750	770	
GTGGCTGTGTTGTTGCAAGTCTTACTGTGGAAGAAAGTC	CTTCCCTAC	GTGAAAGGC	
V A V P V C K S L L W K K V L P Y L K G			
790	810	830	
ATCTGCTCAGGTGGTGGGGAC	CTGAGCGTGTG	ACAGAACGTC	ACACGGACCTGGG
I C S G G G D P E R V D R S S Q R P G			
850	870	890	
GCTGAGGACAATGTCTCAATGACATCGTGA	GTATCTTGCA	GGCCCACCCAG	GTCCCTGAG
A E D N V L N E I V S I L Q P T Q V P E			
910	930	950	
CAGGAAATGGAAGTCCAGGAGCCAGCAGGCCAACAGGTGTC	ACATGTTG	CCCCGGG	
Q E M E V Q E P A E P T G V N M L S P G			
970	990	1010	
GAGTCAGACATCTGCTGGAACGGCAGAACGCTGAAAGGTCTCAGAGGAGGAGGCTGCTG			
E S E H L L E P A E A E R S Q R R R L L			
1030	1050	1070	

Figure 1 (continued)

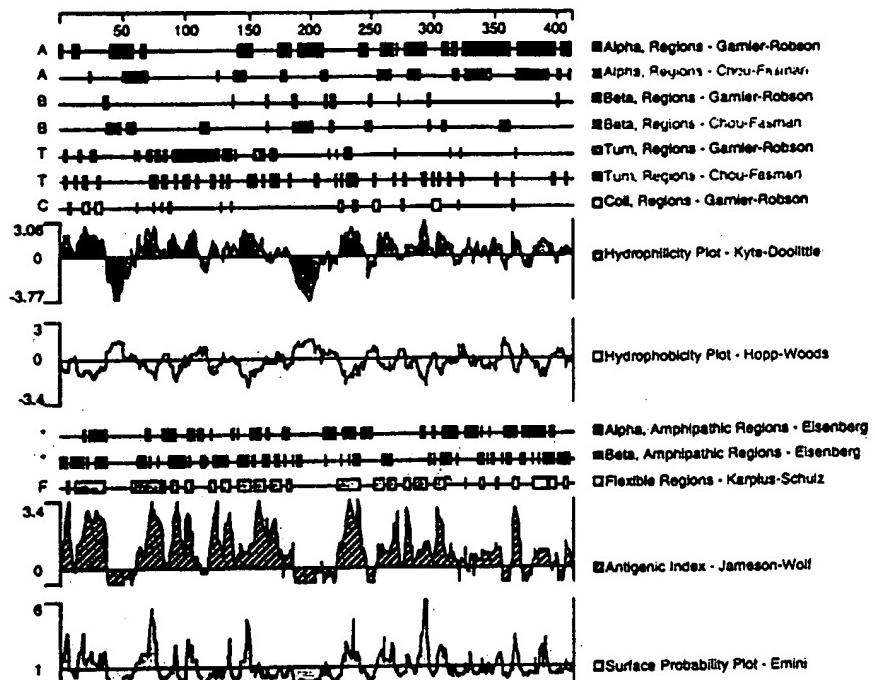
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D L V P F D S W E P L M R K L G L M D N
1150 1170 1190
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E I K V A K A E A A G H R D T L Y T M L
1210 1230 1250
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I K W V N K T G R D A S V H T L L D A L
1270 1290 1310
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E T L G E R L A K Q K I E D H L L S S G
1330 1350 1370
AAGTTCATGTATCTAGAAGGTAAATGCAGACTCTGCCATGTCTTAAGTGTGATTCTCTCA
K F M Y L E G N A D S A M S *
1390 1410 1430
GGAAGTGAACCTTCCCTGGTTTACCTTTTCTGGAAAAAGCCCAACTGGACTCCAGTC
1450 1470 1490
AGTAGGAAAGTGCCACAATTGTCAATGACCGTACTGGAAGAAACTCTCCCATCCAACA
1510 1530 1550
TCACCCAGTGGATGGAACATCCTGTAACCTTCACTGCACTGGCATTATTTTATAAGC
1570 1590
TGAATGTATAAAGGACACTATGGAAAAAAAAAAAAA

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Figure 2

Decoration : Decoration #1: Shaded (with solid black) residues that match the Consensus exactly.

Figure 3



50054021-072997

Figure 4

HAPBU13R

1 AATTCCGGCAC AGCTCTTCAG GAAAGTCAGAC CTTCCCTGGT TTACCTTTTT
51 TCTGGAAAAA GCCCAACTGG GACTCCAGTC AGTAGGAAAG TGCCACAATT
101 GTCACATGAC CGGTACTGGA AGAAAACTCTC CCATCCAACAA TCACCCAGTG
151 GNATGGGAAAC ACTGATGAAC TTTTCACTGC ACTTGGCATT ATTTTTGTNA
201 AGCTGAATGT GATAATAAGG GCACTGATGG AAATGTCCTGG ATCATTCCGG
251 TTGTGCGTAC TTTGAGATTT GNNTTTGGGG ATGTNCATTG TGTTTGACAG
301 CACTTTTTTN ATCCCTTAATG TNAAATGCNT NATTTGATTG TGANTTGGGG
351 GTNAACATTG GTNAAGGNTN CCCNTNTGAC ACAGTAGNTG GTNCCCGACT
401 TANAATNGNN GAANANGATG NATNANGAAC CTTTTTTTGG GTGGGGGGGT
451 NNCGGGGCAG TNNAANGNNG NCTCCCCAGG TTTGGNGTNG CAATNGNGGA
501 ANNNNTGG

HSBBU76R

1 TTTTTTTTGT AGATGGATCT TACAATGTAG CCCAAATAAA TAAATAAAAGC
51 ATTTACATTA GGATAAAAAA GTGCTGTGAA AACAAATGACA TCCCAAACCA
101 AATCTCAAAG TACGCACAAA CGGAATGATC CAGACATTTC CATAGNGTCC
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9004021-076624

Figure 5

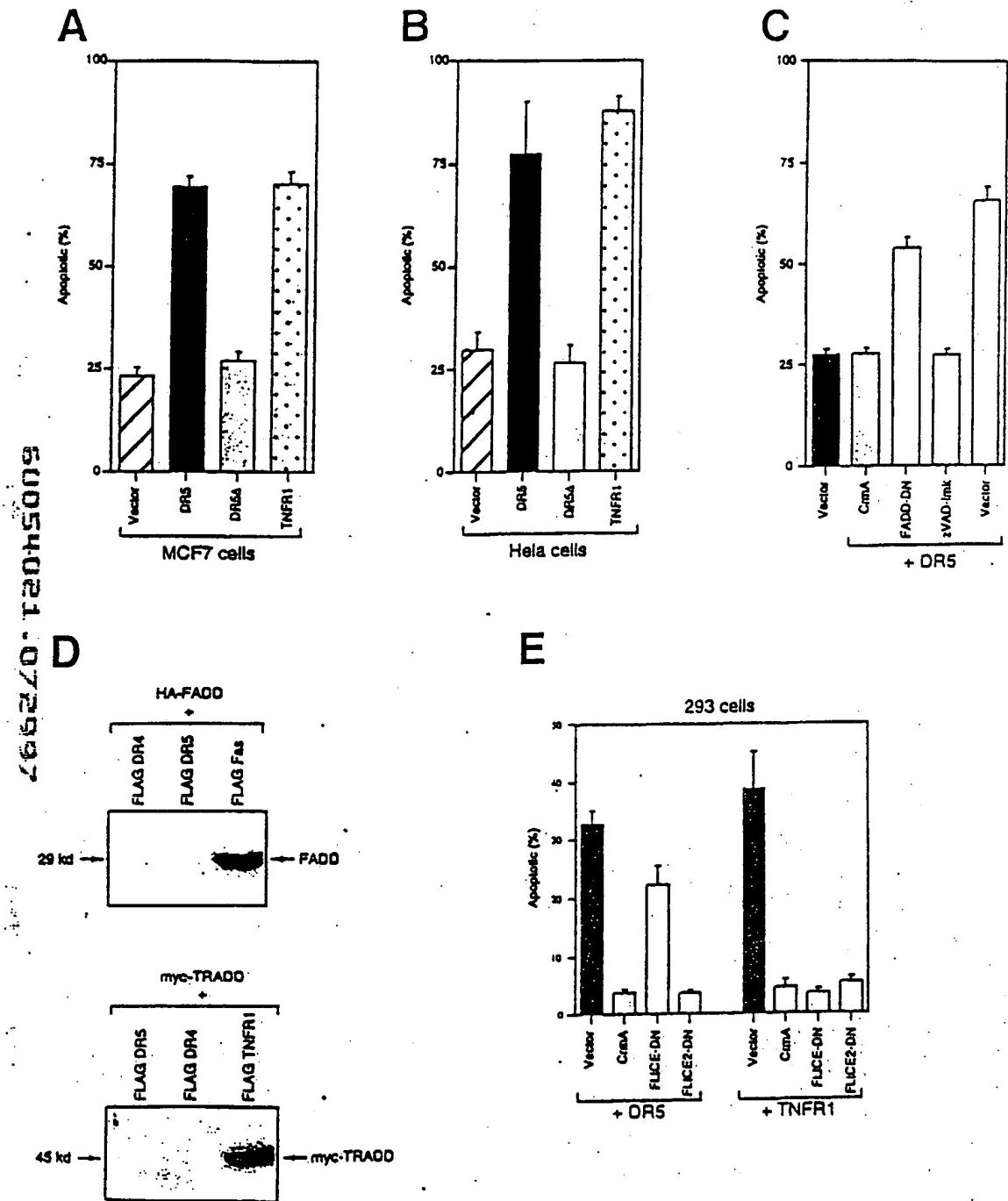
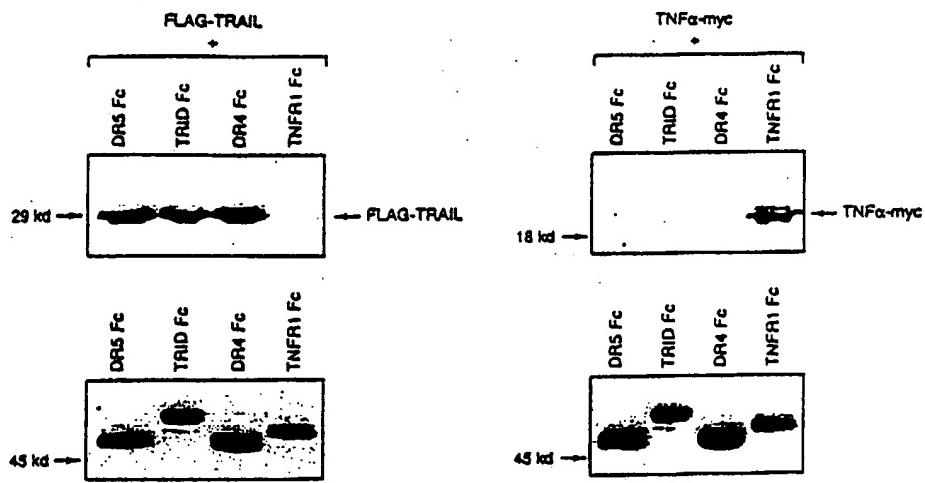
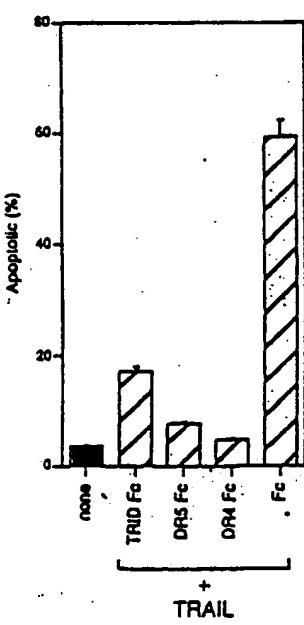


Figure 6

A



B



C

